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THE UNIVERSITY OF ALBERTA

STUDIES OF PLANT FOLYLPOLYGLUTAMATE SYNTHETASE

BY

HELENA CECILE IMESON

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Studies of Plant Folylpolyglutamate Synthetase submitted by Helena Cecile Imeson in partial fulfilment of the requirements for the degree of Master of Science in Plant Biochemistry

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Date: September 29, 1989

TO MY PARENTS

ABSTRACT

Folylpolyglutamate synthetase (FPGS), the enzyme that synthesizes the conjugated folates for one-carbon metabolism, was isolated from pea (Pisum sativum L. cv Homesteader) cotyledons 18 hours after imbibition of dry seeds. Although the folylpolyglutamate pool increases during germination, FPGS activity remained relatively constant. Activity was not affected when seeds were imbibed in cycloheximide, chloramphenicol or methotrexate, suggesting that maintenance of FPGS levels did not require net enzyme synthesis.

Folylpolyglutamate synthetase activity was purified by a protocol involving fractionation of crude extracts by streptomycin sulphate, ammonium sulphate, and chromatography on Sephacryl S-200, DEAE-cellulose and phenylagarose. Protease inhibitors, 2-mercaptoethanol and glycerol were required to stabilize enzyme activity. The enzyme was purified 2700-fold with 40% recovery.

The pea cotyledon enzyme was similar to other eukaryotic synthetases with regard to a), requirements for a folate substrate, MgATP, and L-glutamate; b), a molecular weight of 68,000; and, c), maximal activity at pH 8.5. The polyglutamate products were examined by high performance liquid chromatography after cleavage to p-aminobenzoylpolyglutamates and purification as azo dye derivatives. Tri- and tetraglutamates were the major products when the folate concentration was 10 µM. At 100 µM folate, glutamate was principally incorporated into a diglutamate. Polyglutamate chain lengths were also affected by incubation time.

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LIST OF ABBREVIATIONS

AICAR	5-phosphoribosyl-5-amino-4-imidazole
BSA	bovine serum albumin
μCi	microCurie
CAP	chloramphenicol
CHI	cycloheximide
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
dpm	disintegrations per minute
FPGS	folylpolyglutamate synthetase
GAR	5-phosphoribosylglycinamide
HPLC	high performance liquid chromatography
PteGlu	pteroylglutamic acid (folic acid)
H_4PteGlu	tetrahydropteroylglutamic acid
$H_4PteGlu_n$	H_4PteGlu polyglutamate
5-HCO-H_4PteGlu	N^5-formyl-H_4PteGlu
10-HCO-H_4PteGlu	N^{10}-formyl-H_4PteGlu
5,10-CH=H_4PteGlu	N^5,N^{10}-methenyl-H_4PteGlu
5,10-CH_2-H_4PteGlu	N^5,N^{10}-methylene-H_4PteGlu
5-CH_3-H_4PteGlu	N^5-methyl-H_4PteGlu
$pABAGlu_n$	p-aminobenzoyl polyglutamate
PMSF	phenylmethylsulfonyl fluoride
SAX	strong anionic exchange
SHMT	serine hydroxymethyltransferase
THFA	tetrahydrofolic acid

I. INTRODUCTION

A variety of biosynthetic and degradative reactions involve the addition of one-carbon (C_1) units (Blakley, 1969). Many important cellular components, including purines, pyrimidines, thymidylate, and methionine are produced via the reactions of C_1 metabolism. C_1 metabolism also has a role in bacterial and organelle protein synthesis through the formation of formylmethionyl tRNA. Much work has been done with regard to examining the metabolic pathways within the cell that lead to the formation of these important cellular constituents. The reader's attention is directed to recent reviews on this subject (Cichowicz et al., 1981; Cossins, 1980, 1987; Kisliuk, 1981; Shane and Stokstad, 1985) for further information.

Folates are vitamins that serve as coenzymes in a variety of biological reactions, including the reactions of C_1 metabolism. In these syntheses, one-carbon units are donated by folate derivatives. Knowledge of folate biochemistry has increased dramatically in the last 40 years. Much information is now available on the generation of C_1 units, the biosynthesis of metabolically active folates, and on the control of folate-dependent pathways. Because folates and antifolates play an important clinical role, especially in terms of cancer therapy, much of the original work was done using mammalian systems. Bacterial systems were used for nutritional studies (Blakley, 1969). Investigations of plant folates have, however, increased in the last two decades. A recent book, edited by Blakley

and Benkovic (1984) provides a detailed discussion of present day knowledge of all aspects of folate biochemistry.

The Introduction to this thesis is divided into three sections. First, the biochemistry of C_1 metabolism is discussed, including a review of the compounds donating one-carbon units, as well as the folate-dependent reactions producing cellular constituents. Second, the nature of folic acid and its derivatives is examined. Finally, the synthesis of folylpolyglutamates, the physiologically active form of folate, is reviewed.

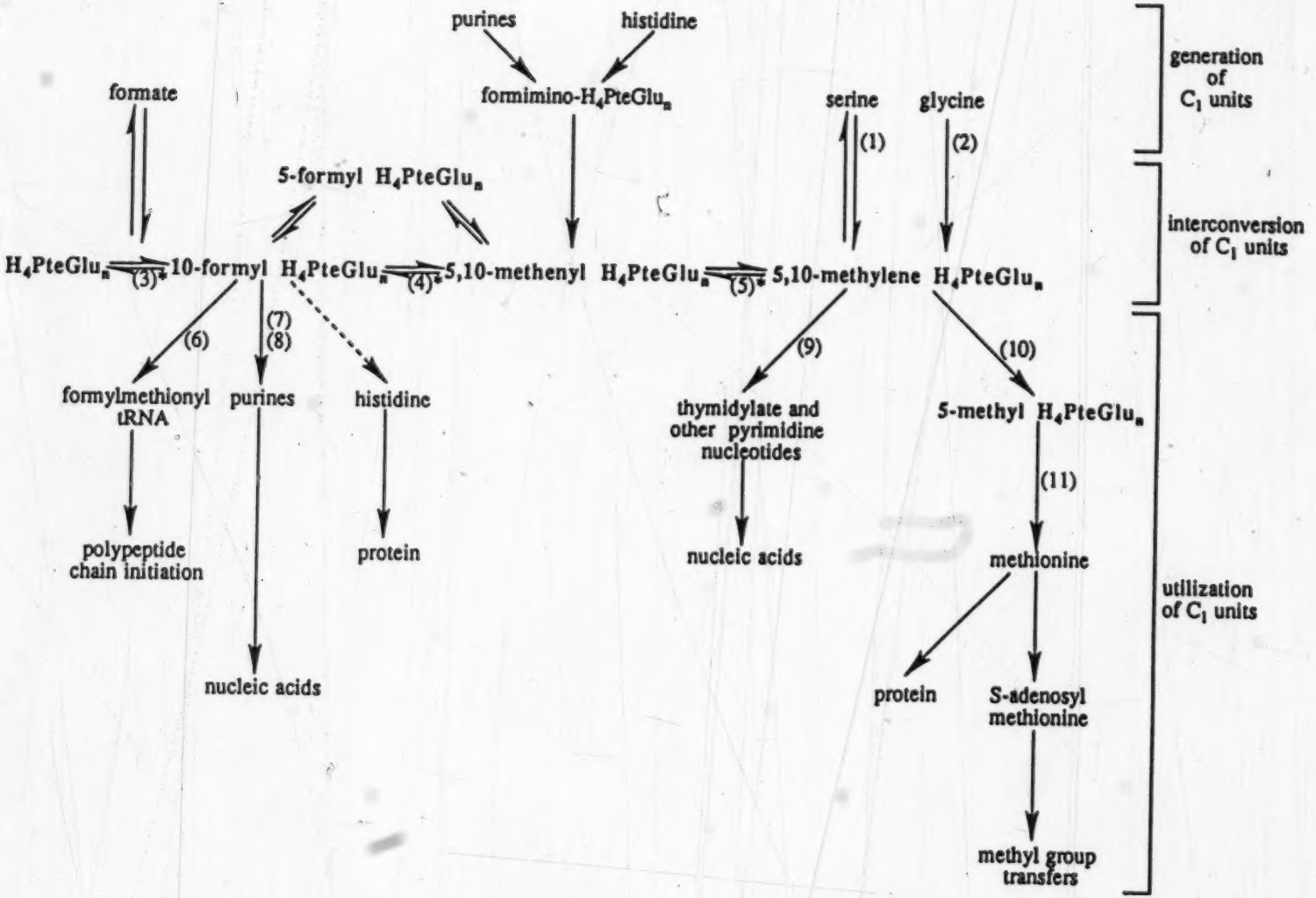
1.1 The Biochemistry of C_1 Metabolism

The series of biochemical reactions leading to the addition of one-carbon units to yield important cellular constituents is referred to as one-carbon metabolism. Many biochemically important compounds, including methionine, formylmethionyl tRNA, purines, pyrimidines, and thymidylate are synthesized in this manner. The one-carbon metabolism of all prokaryotic and eukaryotic cells involves reactions which are folate-dependent, as is shown in Figure 1. These reactions can be divided into three distinct groups: the generation of one-carbon units, the interconversion of folate derivatives, and the incorporation of C_1 units into new compounds. Each of these three groups of reactions is described in more detail in the following paragraphs.

Figure 1. The major pathways of folate-mediated C_1 metabolism.

Reaction #	Enzyme Name	E.C. Number
1.	Serine hydroxymethyltransferase	2.1.2.1
2.	Glycine synthase	2.1.2.10
3.	10-Formyltetrahydrofolate synthetase*	6.3.4.3
4.	5,10-Methenyltetrahydrofolate cyclohydrolase*	3.5.4.9
5.	5,10-Methylenetetrahydrofolate dehydrogenase*	1.5.1.5
6.	Methionyl-tRNA transformylase	2.1.2.9
7.	Glycinamide ribotide (GAR) transformylase	2.1.2.2
8.	Phosphoribosylaminoimidazole-carboxamide (AICAR) transformylase	2.1.2.3
9.	Thymidylate synthase	2.1.1.45
10.	5,10-Methylene tetrahydrofolate reductase	1.1.1.68
11.	Methionine synthase	2.1.1.13

*trifunctional protein referred to as C_1-tetrahydrofolate (C_1-THF) synthase.


purines
histidine
formimino-H4PteGlun
formate
5-formyl H4PteGlun
serine
glycine
(1)
(2)
H4PteGlun
(3)
10-formyl H4PteGlun
(4)
5,10-methenyl H4PteGlun
(5)
5,10-methylene H4PteGlun
(6)
(7)
(8)
(9)
(10)
formylmethionyl tRNA
purines
histidine
thymidylate and other pyrimidine nucleotides
5-methyl H4PteGlun
(11)
polypeptide chain initiation
protein
nucleic acids
nucleic acids
methionine
protein
S-adenosyl methionine
methyl group transfers
generation of C1 units
interconversion of C1 units
utilization of C1 units

One-carbon units are generated from primarily three compounds: serine, glycine and formate (Figure 1). Of these three, serine and glycine are quantitatively the most important contributors (MacKenzie, 1984). Histidine, as indicated in Figure 1, contributes to the one-carbon pool as well, but is not discussed here as its contribution is a minor one. The reader is directed to an excellent review by Shane and Stokstad (1984) for more detailed information on the role of histidine.

Serine and glycine generate 5,10-methylenetetrahydrofolate (5,10-CH_2H_4PteGlu) in reactions catalyzed by serine hydroxymethyltransferase, E.C. 2.1.2.1 (equation 1) and glycine synthase, E.C. 2.1.2.10 (equation 2). Both of these enzymes have been researched

$$\text{Serine} + H_4\text{PteGlu} \rightleftharpoons 5,10\text{-}CH_2H_4\text{PteGlu} + \text{glycine} \qquad (1)$$

$$\text{Glycine} + H_4\text{PteGlu} + \text{NAD} \rightleftharpoons 5,10\text{-}CH_2H_4\text{PteGlu} + \text{NADH} + CO_2 + NH_3 \qquad (2)$$

extensively, and as a result, much information on their regulation and physiological importance has been obtained (Cossins, 1980; Keys, 1980; Schirch, 1982, 1984). Serine hydroxymethyltransferase catalyzes a completely reversible reaction, and, as such, plays a role in the formation of both glycine and serine. Glycine synthase catalyzes the oxidative cleavage of glycine (Kikuchi, 1973). The reactions catalyzed by these two enzymes are coupled in photosynthetic organisms and play a key role in photorespiratory carbon metabolism (Keys, 1980; Tolbert, 1980).

Glycine synthase is a complex of four closely associated proteins designated as the P-, H-, L-, and T- subunits in bacterial cells and in animal mitochondria (Schirch, 1984). Glycine decarboxylation is mediated by the P- and H- subunits, while the T- protein catalyzes the formation of 5,10-CH_2H_4PteGlu. The L- protein mediates the formation of NADH. There is some evidence to suggest that a similar system is present in higher plants. Studies by Sarojini and Oliver (1983) of pea leaf mitochondria suggest the involvement of P- and H- subunit proteins in the decarboxylation of glycine.

Serine hydroxymethyltransferase (SHMT) has been isolated in crystalline form from several animal tissues (Schirch, 1984). It consists of four identical subunits and exists as both cytosolic and mitochondrial isoenzymes. Isoenzymes of SHMT also occur in higher plants (Cossins, 1980). Detailed studies by Rao and Rao (1982) using mung bean seeds as an enzyme source showed the plant enzyme was also composed of four subunits. As well, the physical and catalytic properties of the enzyme were similar to those described for the mammalian enzyme (Schirch, 1984). Mitchell et al. (1986) purified the enzyme over 1500-fold from soybean nodule extracts and determined that it was a tetramer composed of four identical subunits. Neither study used a polyglutamyl folate as a substrate or examined the possibility of inhibition by 5-CH_3H_4PteGlu. Both of these compounds play an important role in the regulation of mammalian SHMT (Matthews et al., 1982).

One-carbon substituted folates may also be generated from formate, as Figure 1 indicates. Formate can arise from glycolate and glyoxylate in plants (Cossins, 1980). Glyoxylate is decarboxylated to give rise to formaldehyde, which is then oxidized to formate (Davies and Corbett, 1969). Equation 3 below outlines the synthesis of 10-formyltetrahydrofolate (10-formylH_4PteGlu) from formate via formyltetrahydrofolate synthetase (E.C. 6.3.4.3). Although this enzyme is present in a wide variety of plants, very few detailed

$$\text{Formate} + \text{ATP} + H_4\text{PteGlu} \rightleftharpoons 10\text{-}HCOH_4\text{PteGlu} + \text{ADP} + P_i \quad (3)$$

studies have been made (Cossins, 1980). However, the plant enzyme has been shown to resemble the corresponding animal and bacterial synthetases in that it is activated by Mg^{2+} and by univalent cations, and that ammonium enhances the binding of formate to the active site. Plant formyltetrahydrofolate synthetase activity may be primarily localized in the cytoplasm (Hiatt, 1965; Iwai et al., 1967; Halliwell, 1973; Crosti, 1974) but there is also good evidence for the presence of similar activity in pea mitochondria (Clandinin and Cossins, 1972) and in the chloroplasts and mitochondria of spinach (Crosti, 1974).

Serine, glycine and formate donate one-carbon units to tetrahydrofolate to form one-carbon substituted tetrahydrofolate derivatives. Organisms must be able to vary the oxidation state of the one-carbon units attached to the folate coenzyme in order to

synthesize the diverse products of C_1 metabolism. For example, if the majority of C_1 units enter the folate pool as 10-formyl derivatives, a mechanism must exist whereby 10-formyl groups are reduced to 5-methyl groups when the cell requires increased levels of methionine. The interconversion between 10-$HCOH_4$PteGlu and 5,10-CH_2H_4PteGlu is carried out by three enzymes: formyltetrahydrofolate synthetase (E.C. 6.3.4.3), which, as discussed above (equation 3) incorporates formate into 10-formyltetrahydrofolate; methylenetetrahydrofolate cyclohydrolase (E.C. 3.5.4.9), which converts 10-$HCOH_4$PteGlu into 5,10-CHH_4PteGlu (equation 4); and methylenetetrahydrofolate dehydrogenase (E.C. 1.5.1.5), which converts 5,10-CHH_4PteGlu to 5,10-CH_2H_4PteGlu (equation 5).

$$10\text{-}HCOH_4PteGlu + H^+ \rightleftharpoons 5,10\text{-}CHH_4PteGlu + H_2O \qquad (4)$$

$$5,10\text{-}CHH_4PteGlu + NADPH \rightleftharpoons 5,10\text{-}CH_2H_4PteGlu + NADP \qquad (5)$$

These three enzyme activities are all completely reversible, allowing for an equilibrium between the pools of C_1 units at the formyl and methylene levels of oxidation.

Because these three enzymes are so important in C_1 metabolism, much work has been done on their physical and catalytic properties. Formyltetrahydrofolate synthetase, methylenetetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase activities are

associated with a single polypeptide in certain eukaryotes (Paukert et al., 1976, 1977; Tan et al., 1977; Caperelli et al., 1980; Schirch, 1978; de Mata and Rabinowitz, 1980). This trifunctional protein has been isolated from mammalian sources, from yeast (MacKenzie, 1984; Appling and Rabinowitz, 1985a), and recently has been characterized from spinach leaves (Nour and Rabinowitz, 1988).

The complex of these three enzyme activities, which have been collectively named C_1-tetrahydrofolate (C_1-THF) synthase (Appling and Rabinowitz, 1985a,b), display a preference for polyglutamated folates (Ross et al., 1984; MacKenzie, 1984; Rabinowitz, 1983). Polyglutamates constitute the bulk of the folate pool (Cossins, 1984) and will be discussed in more detail in the following section. It has been suggested (Appling and Rabinowitz, 1985a) that the collective regulation of this group of enzymes would allow the organism to maintain tight control over C_1 metabolism. One-carbon units could be rapidly diverted in response to a change in cellular demand. However, little research has been carried out in higher plants although this is a very basic area of folate metabolism.

C_1 substituted folates attached to tetrahydrofolate may be utilized in a variety of metabolic reactions (Figure 1). The synthesis of many important cellular constituents, including histidine, formylmethionyl tRNA, purines, thymidylate, and methionine depends on the incorporation of one-carbon units. A brief discussion on the synthesis of each of these compounds follows below.

Formylmethionyl transfer RNA (fMet-tRNA) initiates polypeptide synthesis in bacteria and in the mitochondria and chloroplasts of eukaryotes (Staben and Rabinowitz, 1984). C_1 metabolism is closely linked to protein synthesis as the formyl groups donated to methionyl-tRNA arise from 10-$HCOH_4$PteGlu (equation 6). The enzyme catalyzing this reaction is termed methionyl-tRNA transformylase (E.C. 2.1.2.9). It has been isolated from a variety of organisms, including several higher plant species. Coffin and Cossins (1986)

$$10\text{-}HCOH_4PteGlu + \text{methionyl-tRNA} = \text{formylmethionyl-tRNA} + H_4PteGlu \qquad (6)$$

isolated pea and peanut mitochondrial methionyl-tRNA transformylase activities which showed absolute requirements for 10-formyltetrahydrofolate. Isolated pea mitochondria were able to incorporate [^{3}H]leucine and [^{35}S]methionine into protein in a malate- and ADP- requiring reaction.

Danchin (1973) suggested this reaction could regulate the synthesis of both RNA and protein by controlling the levels of 10-$HCOH_4$PteGlu in bacteria. Direct evidence has also been collected implicating formylmethionyl-tRNA in eukaryotic protein synthesis. Polypeptide synthesis in yeast mitochondria and Euglena chloroplasts (Bianchetti et al., 1977; Lucchini and Bianchetti, 1980) was stimulated by the addition of 10-$HCOH_4$PteGlu but ceased rapidly when this source of C_1 units was absent. As well, the addition of 5-$HCOH_4$PteGlu, which competitively inhibits methionyl-tRNA

transformylase, strongly inhibited protein synthesis, indicating that a specific folate-dependent C_1 transfer reaction was of central importance in this process (Lucchini and Bianchetti, 1980).

10-formyltetrahydrofolate is required in the biosynthesis of purines. It serves as a precursor of C-2 and C-8 in the purine ring (Rowe, 1984). These folate-dependent reactions (equations 7 and 8) are catalyzed by two specific enzymes: glycinamide ribotide (GAR)

5-phosphoribosylglycinamide (GAR) + 10-$HCOH_4$PteGlu ⟶
5-phosphoribosyl-N-formylglycinamide + H_4PteGlu (7)

5-phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR) + 10-$HCOH_4$PteGlu ⟶ 5-phosphoribosyl-5-formamido-4-imidazole carboxamide + H_4PteGlu (8)

transformylase (E.C. 2.1.2.2) and phosphoribosylaminoimidazole-carboxamide (AICAR) transformylase (E.C. 2.1.2.3). Both enzymes display a preference for polyglutamates of 10-$HCOH_4$PteGlu (Chan and Baggott, 1982; Rowe, 1984). The GAR transformylase has not been characterized from a higher plant source, although there is evidence for the AICAR transformylase in a variety of plant species (Iwai et al., 1972).

The GAR and AICAR transformylases are associated with the tri-functional C_1-THF synthase and serine hydroxymethyltransferase in avian livers (Caperelli et al., 1980). This multienzyme complex could have physiological significance with regard to channelling C_1 units originating from serine through the methylene, methenyl, and formyl oxidation levels to final utilization in the GAR and AICAR transformylation reactions. 10-formyltetrahydrofolate is also involved in the synthesis of histidine, as it indirectly supplies C-2 of the histidine ring. 10-$HCOH_4$PteGlu acts as a substrate for the synthesis of the purine ring, which in turn is the direct precursor of C-2 of the histidine ring (Shane and Stokstad, 1984).

Folate derivatives play an important role in the biosynthesis of nucleic acids. C_1 units are donated by 5,10-CH_2H_4PteGlu to deoxyuridine monophosphate, forming thymidylate (equation 9). The reaction is catalyzed by thymidylate synthase (E.C. 2.1.1.45). Di-

$$\text{deoxyuridine monophosphate} + 5,10\text{-}CH_2H_4\text{PteGlu}_n \longrightarrow \text{thymidine monophosphate} + H_2\text{PteGlu}_n \qquad (9)$$

hydrofolate is produced in this reaction, as the 6-hydrogen from the pteridine ring of H_4PteGlu is incorporated into thymidylate. Dihydrofolate reductase, which catalyzes the conversion of dihydrofolate to tetrahydrofolate, must therefore be present in order to maintain thymidylate synthesis.

Thymidylate synthase has been isolated and examined in bacterial wild-type and mutant strains as well as in higher organisms (Santi and Danenberg, 1984). Algae and green plants have not received as much attention (Feller et al., 1980; Bachmann et al., 1983; Vandiver and Fites, 1979) although a recent study of thymidylate synthase in carrot cell suspension cultures has recently been published (Nielsen and Cella, 1988). In the majority of eukaryotic cells, thymidylate synthase is low and may therefore limit the rate of DNA replication (Reddy, 1982; Grobner and Loidl, 1983). Maximal enzyme activity was achieved immediately before or during the S-phase of the cell-cycle in cultured cells. Most thymidylate synthases studied show a preference for polyglutamate derivatives.

Methylenetetrahydrofolate reductase (E.C. 1.1.1.68) catalyzes the reduction of 5,10-methyleneH_4PteGlu to 5-methylH_4PteGlu (equation

$$5,10\text{-}CH_2H_4PteGlu + FADH_2 \rightleftharpoons 5\text{-}CH_3H_4PteGlu + FAD \qquad (10)$$

10). This reaction is physiologically irreversible and represents the only route for <u>de novo</u> formation of the methyl group of methionine (Matthews, 1984). C_1 units at this level of reduction are committed to methionine synthesis as there are no other known metabolic fates for this derivative. The terminal biosynthetic reaction producing methionine in plants is catalyzed by a cobalamin-independent methionine synthase, E.C. 2.1.1.13 (equation 11). The

corresponding enzymes in mammalian cells and in some bacteria are

$$5\text{-}CH_3H_4PteGlu_n + \text{L-homocysteine} \longrightarrow \text{L-methionine} + H_4PteGlu_n \quad (11)$$

cobalamin-dependent synthases (Matthews, 1984). Cobalamin-independent methionine synthases have a strict requirement for polyglutamyl forms of $5\text{-}CH_3H_4PteGlu$. For example, the K-12 strain of E. coli will not methylate homocysteine in the presence of $5\text{-}CH_3H_4PteGlu$ (Whitfield et al., 1970). Detailed studies of the biosynthetic pathway leading to methionine in higher plants have been carried out (Giovanelli et al., 1980, 1985; Thompson et al., 1982) but they have tended not to examine the generation of methylated folate or the final transmethylation reaction (Cossins, 1987).

1.2 The Nature of the Folate Molecule

Pteroylglutamic acid (PteGlu) and its related derivatives are normal constituents of all living cells (Stokstad, 1943; Pfiffner et al., 1943, 1946; Keresztesy et al., 1943; Day et al., 1945; Hutchings et al., 1944; Totter et al., 1944). Folate was identified as an antianemia agent in animals (Wills, 1931; Hogan and Parrott, 1940), and as a bacterial growth factor (Snell and Peterson, 1940; Mitchell et al., 1941; Stokstad, 1943; Keresztesy et al., 1943). Mitchell and his group (Mitchell et al., 1941, 1944; Frieden et al., 1944; Mitchell and Williams, 1944; Mitchell, 1944), using spinach leaves as a source of the vitamin, were the first to isolate, characterize, and purify the compound, which they termed folic acid.

Microbiological assays were then developed, allowing for the examination of many different tissues for the presence of folic acid and related derivatives.

Microbiological assays are based on the fact that folic acid and its derivatives stimulate the growth of Lactobacillus casei (ATCC 7469), Streptococcus faecalis (ATCC 8043) and Pediococcus cerevisiae (ATCC 8081) (Blakley, 1969). This growth response is specific, as S. faecalis does not respond to methylated derivatives and P. cerevisiae responds only to highly reduced, but not methylated derivatives. L. casei allows for the assay of all naturally occurring folates when oxidation is prevented by the addition of ascorbate (Bakerman, 1961). Polyglutamyl folates must be hydrolyzed before assay (Roos and Cossins, 1971). Each of the three organisms gives very little growth in the absence of folate. Linear increases in the growth rate occur if 0.2 to 1.0 ng of folate is present (Spronk, 1971). These sensitive assays can therefore be used to obtain information on the type and amount of folate present in different extracts.

Radioassay techniques have also been applied to folate analyses (for review, see Cossins, 1984). In this method, both labelled and unlabelled folates compete for binding sites on folate-binding proteins. This method has been used extensively in the analysis of serum and red blood cell folates and results in data that agree with those obtained by microbiological assay. This method has not been employed in assays of plant folates.

Folate derivatives show differences in C_1 unit substitution, degree of oxidation, and glutamyl conjugation (Blakley, 1969; Cossins, 1980). Folates can now be isolated from one another by taking advantage of these differences. A variety of chromatographic techniques, including ion exchange, gel filtration, and high performance liquid chromatography (HPLC) have been successfully applied. Chemical cleavage of folate derivatives to p-aminobenzoyl-polyglutamates ($pABAGlu_n$), followed by HPLC is a rapid and effective method for the analysis of naturally occurring folates (Cossins, 1984). Cleavage of folate polyglutamates occurs at the C-9-N-10 bond, yielding a series of p-aminobenzoylpolyglutamates (Baugh et al., 1979; Foo et al., 1980, Shane, 1986). After concentration, these can be detected by HPLC.

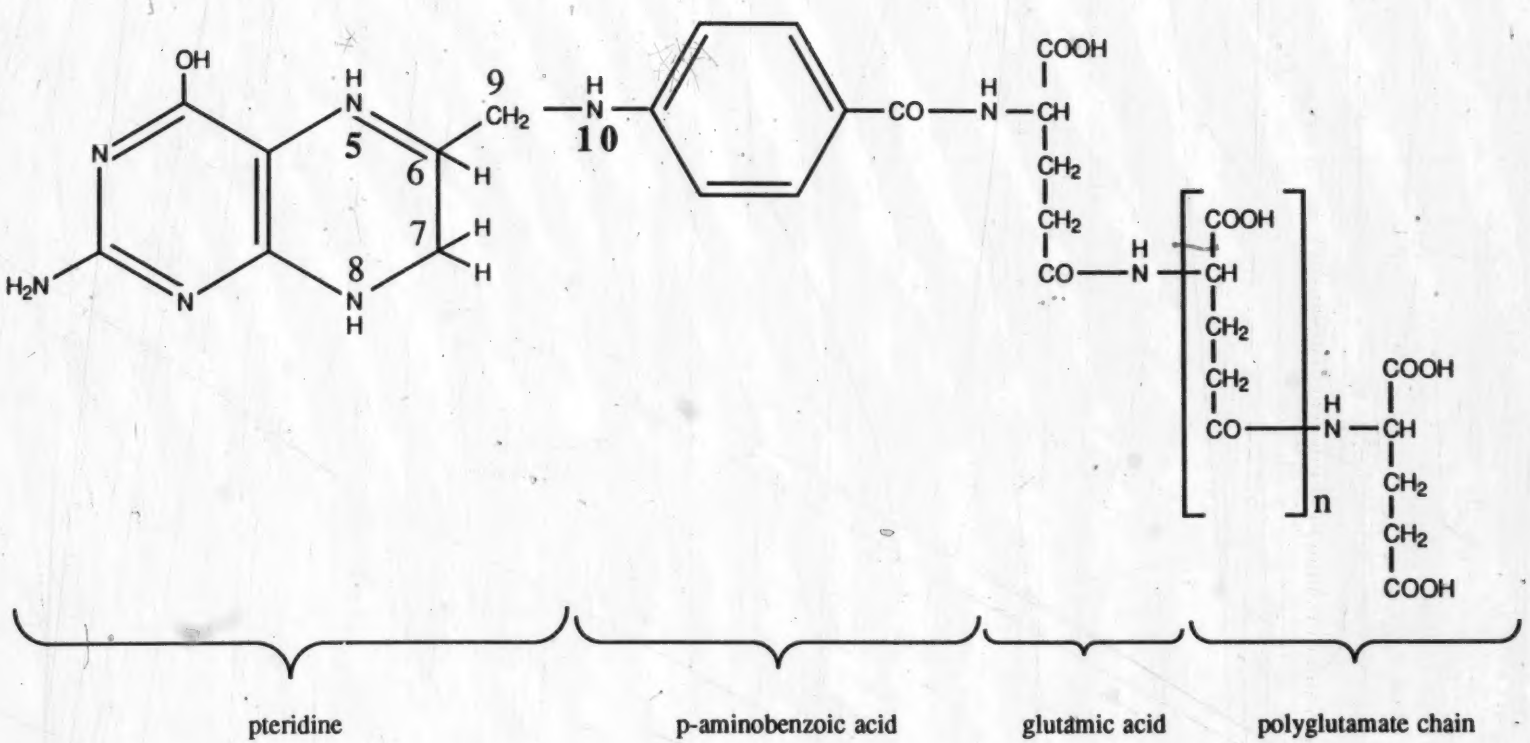
Folates are made up of three structural components; a pteridine ring, p-aminobenzoic acid, and one or more L-glutamic acids (Figure 2). A complex pathway is responsible for the biosynthesis of tetrahydrofolate. The steps and enzymes involved in this pathway are briefly described in the following paragraph.

In bacteria and plants folate synthesis commences with the formation of the pteridine component from guanosine triphosphate (Blakley, 1969; Brown, 1970; Mitsuda et al., 1966). Hydroxymethyldi-hydropteridine, the pteridine derivative formed from guanosine triphosphate, is converted to dihydropteroic acid in plants (Iwai et al., 1968; Iwai and Okinaka, 1968; Ikeda and Iwai, 1970) and E. coli (Brown, 1970) in a two step reaction catalyzed by dihydropteroate synthase. Dihydrofolate synthetase catalyzes the bonding of

FIGURE 2

STRUCTURE OF TETRAHYDROFOLIC ACID.

In metabolically important folates, C_1 substitution occurs at N^5 to give 5-formyl and 5-methyl derivatives and at N^{10} to give 10-formyl derivatives. Native folates are primarily polyglutamyl compounds generally containing 3-8 gamma-glutamyl residues.


pteridine
p-aminobenzoic acid
glutamic acid
polyglutamate chain

L-glutamic acid to dihydropteroate in an ATP- and Mg^{2+}- requiring reaction to form dihydrofolate. Dihydrofolate synthetase activity has been detected in nine different plant species (Ikeda and Iwai, 1970). Dihydrofolic acid is then reduced to tetrahydrofolic acid by dihydrofolate reductase. This enzyme has been purified from both mammalian and bacterial systems (Blakley, 1969; Brown, 1970) and from plant sources (Suzuki and Iwai, 1970; Reddy and Rao, 1976, Ratnam et al., 1987).

The final step in the synthesis of metabolically active tetrahydrofolate involves the addition of one or more L-glutamic acids in a reaction catalyzed by folylpolyglutamate synthetase (abbreviated as FPGS; equation 12). With the exception of folylpolyglutamate-deficient mutants, all organisms have the capacity

$$H_4PteGlu_n + ATP + \text{L-glutamate} \rightarrow H_4PteGlu_{n+1} + ADP + P_i \quad (12)$$

to synthesize folylpolyglutamates by this reaction, including organisms which are unable to synthesize folic acid. Intracellular folates generally have a polyglutamate chain (attached via gamma linkages) as opposed to a single glutamate residue. The length of this chain normally varies from two to eight residues in most of the species examined, although chain lengths up to twelve have been isolated (Nakamura and Kozloff, 1978). Usually a distribution of lengths centered around a predominant length is found in each species. For example, E. coli contains primarily pentaglutamates,

but tetra- and hexaglutamates are also present. Heptaglutamates are the predominant folates in yeast but significant amounts of hexa- and octaglutamates are also found (Bassett et al., 1976). Mammalian cells contain primarily pentaglutamates (Scott and Weir, 1976). Clostridium is the only known exception to this pattern, as only triglutamates have been isolated (Curthoys et al., 1972). The following section reviews the biosynthesis and functions of folylpolyglutamates.

1.3 The Biosynthesis of Folylpolyglutamates

Folylpolyglutamates are synthesized from tetrahydrofolate monoglutamate and L-glutamate in a reaction that requires Mg^{2+} and ATP. This synthesis is catalyzed by folylpolyglutamate synthetase (equation 12 above). The polyglutamates produced by this reaction have a number of physiologically important roles including: acting as substrates in the reactions of C_1 metabolism; acting as inhibitors, or regulators of C_1 metabolism; and serving as storage forms of the vitamin. In addition, they are required for specialized cellular reactions, including T-even bacteriophage tail assembly in E. coli. Each of these functions is reviewed briefly in the following paragraphs.

Folates act as coenzymes in the folate-dependent reactions of one-carbon metabolism. Studies of the enzymes catalyzing these reactions have illustrated that folylpolyglutamates act as substrates for many or all of them (reviewed by McGuire and Bertino, 1981). The ratio V_{max}/K_m can be used to analyze the relative effectiveness

of polyglutamate and monoglutamate substrates (Baggott and Krumdieck, 1979). This ratio is generally the same or higher when polyglutamates are used, indicating that in most cases they are the preferred substrates. The highest ratio is often obtained with the length of polyglutamate chain which predominates in the organism serving as the enzyme source.

Several mechanisms may explain why polyglutamates are preferentially used as substrates. The polyglutamate chain can be used for increasing the binding to the active site, resulting in a lower K_m but an unaffected V_{max}. The polyglutamate chain may cause a conformational change in the active site, leading to a change in V_{max}. Channelling of folylpolyglutamates through multifunctional proteins like C_1-THF synthase may also lead to increased activity. Reviews by McGuire and Bertino (1981), Kisliuk (1981), Scrimgeour (1986), and McGuire and Coward (1984) provide further information on this subject.

Folylpolyglutamates can inhibit folate dependent reactions for which they are not substrates. It has been hypothesized that folate metabolism could be regulated to some extent by the relative amounts of both substrate and inhibitor present (Kisliuk et al., 1974; Matthews and Baugh, 1980; Krumdieck et al., 1977). McGuire and Bertino (1981) give examples whereby enzymes of C_1 metabolism can be inhibited by a non-substrate folylpolyglutamate present in physiological concentrations. Although it is difficult to specifically inhibit only one enzyme in such a system of close interaction and interconverting forms, there are regulatory

mechanisms of this type found in other complex enzyme systems like glycolysis (Bertino and Hillcoat, 1968). Therefore, such a system may exist in the regulation of the enzymes of one-carbon metabolism.

Folylpolyglutamates were initially detected because the folates extracted from yeast would not serve as growth factors for folate requiring bacteria. Cleavage of these folate compounds by gamma-glutamyl hydrolases to monoglutamate forms was required to make the vitamin available as a growth factor. As well, monoglutamates were shown to be active in newly discovered folate-dependent enzyme reactions. Based on this information, it was commonly assumed that polyglutamates functioned only as inert storage forms of the vitamin. It is now known that hydrolysis of the polyglutamate prior to bacterial utilization was a result of the ineffective transport of these forms by the bacteria, as opposed to their not being utilized as coenzymes in folate-requiring enzymes.

There is recent evidence to suggest that folate may be stored as polyglutamate (Moran et al., 1976). In these studies, only folyl-polyglutamates were present in L1210 cells grown on optimal and suboptimal folate. Cells deprived of folate (0.76 pmol intracellular folate/10^6 cells) grew at 88% the rate of folate sufficient cells (5.6 pmol/10^6 cells). A sevenfold decrease in the folate pool caused only a 12% decrease in growth rate with no apparent change in the relative distribution within the pool. These studies suggest that L1210 cells had a folylpolyglutamate pool in excess of that required for growth. The polyglutamates were either metabolically inactive or were used at a reduced flux rate per molecule.

The effect of polyglutamate formation on the retention of folates and antifolates has also been examined. Most of the studies completed to this date have utilized the anticancer agent methotrexate (McGuire and Coward, 1984). Polyglutamylation of methotrexate leads to a concentration of the drug inside the cell which is considerably higher than that found outside the cell. Free methotrexate effluxes from the cell much more quickly than the polyglutamated form (Galivan et al., 1982). The resultant intracellular retention is the major feature of the chemotherapeutic action of this drug. Studies using human fibroblasts (Foo et al., 1982) and Chinese hampster ovary cells (McBurney and Whitmore, 1974; Foo and Shane, 1982) have shown that polyglutamylation leads to considerably higher concentrations of folylpolyglutamates in the cell as compared to the monoglutamates in the external medium. Polyglutamylation is believed therefore to aid in the intracellular retention of folates.

Folylpolyglutamates are thought to have a variety of highly specialized functions. For example, six molecules of $H_2PteGlu_6$ are required to assemble the tail section of T-even bacteriophage of Escherichia coli (Kozloff, 1980). They are also believed to have effects on other enzymes, to affect enzyme stabilization and enzyme kinetic constants, and they may play a role as allosteric effectors of non-folate reactions. These possible functions of folylpolyglutamates have been reviewed by McGuire and Bertino (1984).

Folylpolyglutamates are the intracellular form of folate. The intracellular polyglutamate chain length is always longer than the chain lengths of the folates transported into and out of cells. All cells must therefore possess a mechanism allowing for the polyglutamylation, and thus, retention of the folates entering them.

The number of enzymes catalyzing the production of the glutamate chain has been examined. Several enzymes would be required if each one was responsible for the addition of only one L-glutamic acid in the polyglutamate chain. Many enzymes would also be needed if each one could only synthesize a polyglutamate chain for a particular one-carbon substituted derivative. However, evidence to date suggests that a single enzyme is involved, either by catalyzing the synthesis of all lengths of all folates, or by catalyzing the synthesis of all lengths of one substituted folate, which could then be converted to other folate derivatives.

McBurney and Whitmore (1974), using Chinese hampster ovary (CHO) cell cultures, have collected data which points to the involvement of a single enzyme. They isolated a mutant cell line incapable of synthesizing folylpolyglutamates and studied the reversion frequency back to the wild-type, which produced pentaglutamates. Reversion frequency was consistent with a single genetic lesion. Biochemical studies by Taylor and Hanna (1977, 1979) also provide strong evidence for the presence of only one enzyme. Crude and twenty five-fold purified folylpolyglutamate synthetase (FPGS) extracts from CHO cells synthesized di- and longer lengths of tetrahydrofolate. Purified FPGS from CHO cells also synthesized polyglutamates from a number of substituted monoglutamyl folates (Taylor and Hanna, 1977). Examin-

ation of extracts of the mutant cell line showed that this line had lost the ability to synthesize diglutamates and longer chain lengths for any folate derivative. This loss was not due to the presence of conjugases or nondialyzable inhibitors. Spontaneous revertants regained the ability to synthesize polyglutamate chains for all derivatives, but the folylpolyglutamate synthetase in these cells had slightly different properties, indicating that the original mutation had occurred in the structural gene for FPGS as opposed to a regulatory gene (Taylor and Hanna, 1979). These studies thus provide evidence for a single folylpolyglutamate synthetase being responsible for the synthesis of all folylpolyglutamates up to pentaglutamates in Chinese hampster ovary cells.

Other evidence from various organisms also points to the involvement of a single enzyme in the synthesis of folylpolyglutamates. Partially purified rat liver folylpolyglutamate synthetase was shown to synthesize pentaglutamate folates, the predominant length found in vivo, from monoglutamates. As well, all folylmonoglutamates had similar pH optimas for activity (McGuire et al., 1980). Comparisons of crude extracts versus 7000-fold purified folylpolyglutamate synthetase using monoglutamate and polyglutamate substrates showed that the relative activities were nearly the same in Corynebacterium, indicating that again, only one enzyme was involved (Shane, 1980). Ferone and Warskow (1983) have now shown that long chain polyglutamates can be synthesized in E. coli, contradicting initial studies by Masurekar and Brown in 1975.

Masurekar and Brown indicated that more than one synthetase might be present, as the enzyme they isolated only synthesized diglutamates of its preferred substrate.

There is evidence for multiple synthetase activities in Neurospora crassa (Ritari et al., 1973; Cossins and Chan, 1983). Ammonium sulphate fractionation and genetic evidence both indicate the presence of two activities: a diglutamate synthetase and another synthetase catalyzing the formation of longer polyglutamates. Shane and his group (Shane 1980; Cichowicz et al., 1981) were unable to repeat the separation of the two activities by ammonium sulphate fractionation as Ritari et al. (1973) had done. Cossins' group is presently attempting to definitively answer this question by making use of the MAC and MET6 nutritional mutants.

Investigations of folylpolyglutamate synthetase in cell free systems have been attempted for several organisms (Table 1). Progress has been slow because the enzyme is present in low amounts, simple assays for activity have only recently been developed, and finally, it is very unstable. Each of these problems is discussed in more detail in the following paragraphs.

Although the enzymes examined show very low amounts of activity, prokaryotes generally exhibit 50-100 times more activity than eukaryotes. This difference in total enzyme activity between pro- and eukaryotes is not reflected in the size of the folylpolyglutamate pool, as both classes are the same order of magnitude. Calculations by Moran and Colman (1984) using folylpolyglutamate synthetase from mouse liver led them to estimate that 100 grams of tissue would be

Table 1. Chronological review of folylpolyglutamate synthetases isolated and purified from various sources.

Source	Purification	Reference
E.coli	n.d.*	Griffin and Brown, 1964
Sheep liver	n.d.	Gawthorne and Smith, 1973
E. coli	75-fold	Masurekar and Brown, 1975
CHO cells**	25-fold	Taylor and Hanna, 1977
Rat liver	55-fold	McGuire et al., 1980
Corynebacterium	6994-fold***	Shane, 1980
Hog liver	200-fold	Cichowicz et al., 1981
L. casei	197,000-fold***	Bognar and Shane, 1983
Mouse liver	300-350-fold	Moran and Colman, 1984
Beef liver	59-170-fold	Pristupa et al., 1984
E. coli (transformant)	93-fold	Bognar et al., 1985
Pea cotyledon	n.d.	Chan et al., 1986
Hog liver	31,500-fold***	Cichowicz and Shane, 1987
Human liver	4-8-fold	Clarke and Waxman, 1987

*n.d. not determined
**Chinese hampster ovary cells
***purified to homogeneity

required to yield 10 micrograms of enzyme. Their calculation quantifying the amount of enzyme in mouse liver shows that it is indeed present in very low amounts.

The distribution of folylpolyglutamate synthetase in mammalian tissues has been examined in rats using assay conditions optimal for the liver enzyme (McGuire et al., 1979). The highest specific activity was found in the liver, which also contained the highest amount of total folate (Richardson et al., 1979). Activity was also detected in the brain, stomach, small intestine, lung and spleen but not in the heart, kidney or other muscle tissue. Heart and muscle tissue contain very low amounts of folylpolyglutamates; hence one would not expect to find synthetase activity. However, kidneys are rich in folates. The apparent lack of activity in this organ could be explained by non-optimal assay conditions or hydrolysis of polyglutamate products by hydrolases.

The subcellular location of folylpolyglutamate synthetase has been investigated using rat liver as an enzyme source (McGuire et al., 1979). The highest specific activity was found in the cytosol (75% of total activity) followed by the nuclear, then the mitochondrial/lysosomal and microsomal fractions. These latter fractions all had about the same specific activity. The high activity in the nuclear fraction is probably due to contamination by the cytosol during the fractionation process. The mitochondrial fraction probably has a higher specific activity than these experiments indicate. Mitochondria contain folylpolyglutamates and presumably must have their own synthetase. One explanation for the low specific activity

involves substrate specificity (McGuire and Bertino, 1981). The mitochondrial synthetase has a different substrate specificity as compared to the cytosolic enzyme. Assay conditions optimal for the cytosolic enzyme would thus be suboptimal for the mitochondrial synthetase.

One of the major obstacles to the study of this enzyme has been the lack of a rapid, simple and sensitive assay. Several methods have been attempted but it is only recently that an assay meeting all of the above criteria has been developed. Initial studies of the enzyme from E. coli used electrophoretic separation of reactants and products followed by bioautography (Griffin and Brown, 1964). Gawthorne and Smith (1973) assayed the synthetase from sheep liver by gel-sieving chromatography, which separated folylmonoglutamates from the polyglutamate products but was very time consuming. Other assays made use of labelled glutamate and charcoal absorption (Masurekar and Brown, 1975; Ritari et al., 1975). In this method, folates with ligated labelled glutamates were absorbed on charcoal. Free glutamate was removed by washing and polyglutamates were eluted with ethanolic ammonia. This assay proved to be unsatisfactory because product elution was not always quantitative and blank values were high. Brody and Stokstad (1982) also measured the incorporation of labelled glutamate but after the incubation, folates were cleaved to p-aminobenzoylpolyglutamates. These compounds were then converted to azo dye derivatives, which could be separated from free glutamate by adsorption chromatography. However, not all folate derivatives were cleaved by the Zn/HCl treatment, making this method unquantifiable.

Priest et al. (1981) have developed an assay based on the incorporation of reaction products into covalent ternary complexes with [^{3}H]5-fluoro-dUMP and thymidylate synthase. The electrophoretic mobility of these complexes was compared with standard complexes prepared from synthetic polyglutamates. Today, the most commonly used assay relies on the separation of incorporated labelled glutamate from free glutamate by DEAE cellulose. This method was developed simultaneously by Taylor and Hanna (1977) and McGuire et al. (1979, 1980).

Crude extracts containing folylpolyglutamate synthetase activity may also contain other enzymes which could interfere with the assay. Enzymes metabolizing glutamate, folate, or ATP may be present as well as gamma-glutamyl hydrolases (conjugases) which will degrade polyglutamate products as they form. Thus it is very important to set up assays which lack either the folate substrate or synthetase activity in order to account for any effects shown by the interfering enzymes.

The last factor which makes the study of the synthetase difficult is its instability. Because of this, one of the first steps in the purification of the enzyme is the elucidation of conditions which will stabilize relatively crude extracts. Storage of extracts at 2^{o}C in phosphate buffer at pH 7.5 with 5 mM mercaptoethanol appears to stabilize mammalian synthetases (Pristupa et al., 1984). More highly purified extracts also require these conditions as well to prevent loss of activity. Cichowicz and Shane, in their 1987 paper on the purification of the synthetase from hog liver, routinely included benzamidine and other protease inhibitors. Triton X-100 was

added as the enzyme appeared to be hydrophobic (Shane, personal communication, 1988). All folylpolyglutamate synthetases examined to date require stabilization prior to and during purification.

As described earlier, investigations of folylpolyglutamate synthetase have been hampered because the enzyme is present in low amounts, is highly unstable, and simple, rapid, and sensitive assays have only recently been developed. Despite these problems, a few synthetases have been isolated and purified (Table 1). The following paragraph presents a chronological overview of the synthetases studied to date. The physical and catalytic properties of these enzymes will be described in the Discussion section of this thesis.

Griffin and Brown (1964) were the first to describe the presence of an enzyme catalyzing the addition of glutamate onto tetrahydrofolate. This enzyme, later termed folylpolyglutamate synthetase, was isolated by Gawthorne and Smith (1973), who studied the *in vitro* synthesis of folylpolyglutamates from sheep liver extracts. The enzyme from *Escherichia coli* was purified 75-fold in 1975 (Masurekar and Brown, 1975), while ten years later, *E. coli* transformants (*E.coli* SF4/B2) were purified to homogeneity (Bognar et al., 1985). Taylor and Hanna (1977) purified the enzyme 25-fold from Chinese hampster ovary cell cultures while developing the assay for activity which is most commonly used today. The synthetase from *Corynebacterium* was the first to be purified to homogeneity (Shane, 1980), and the *Lactobacillus casei* enzyme soon followed (Bognar and Shane, 1983). Several mammalian enzymes have been partially purified in the last ten years, including enzymes from: rat liver, purified

55-fold (McGuire et al., 1980); hog liver, purified 200-fold (Cichowicz et al., 1981); beef liver, purified 59 to 170-fold (Pristupa et al., 1984); and mouse liver, purified 300 to 350-fold (Moran and Colman, 1984). Cichowicz and Shane (1987) are the only researchers to have purified folylpolyglutamate synthetase to homogeneity from a mammalian source (hog liver). The FPGS in Neurospora has been examined by Cossins and Chan (1984) and has recently been purified about 4000-fold from wild-type cultures (Chan et al., 1989).

1.4 The Present Study

As mentioned in earlier sections, folylpolyglutamates are the physiologically active form of folate. They participate in the reactions of one-carbon metabolism, leading to the synthesis of important cellular compounds, including methionine, thymidylate, and purines. Folylpolyglutamates are in turn synthesized from tetrahydrofolate and L-glutamic acid(s) in an ATP- requiring reaction catalyzed by folylpolyglutamate synthetase. This enzyme has proved to be extremely difficult to study, as it is unstable and is present in low amounts. It is only recently that rapid, simple and sensitive assays for activity have developed. Because of these problems, only a few synthetases have been purified and characterized (Table 1). None of the synthetases purified to date have been isolated from a higher plant source, a fact that forms the basis for the research presented in this thesis.

Polyglutamate synthesis has been studied *in vivo* using radish (Spronk 1971) and pea cotyledons during germination (Roos and Cossins, 1971; Chan et al., 1986). Microbiological assays using *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae* showed that a net synthesis of folylpolyglutamates occurred during germination. The latter study (Chan et al., 1986) is the only one found in the literature which examines the *in vitro* synthesis of these compounds in green plants. Both crude and ammonium sulphate extracts synthesized folylpolyglutamates, indicating that folylpolyglutamate synthetase activity was present.

The present study has determined that pea cotyledons are an excellent source of the plant enzyme. Folylpolyglutamate synthetase has been purified and its properties have been examined. These properties have been compared to properties obtained from other FPGS enzymes and conclusions regarding similarities and differences between the enzymes from plant, animal, bacterial and fungal sources were reached.

II. MATERIALS AND METHODS

2.1 Chemicals

Reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis), Fisher Scientific, BDH Chemicals, and Terochem, all from Edmonton, Alberta. Cellex D cellulose was supplied by Bio-Rad Laboratories, Richmond, California, while DE-52 cellulose was supplied by Whatman, Clifton, New Jersey. Sephacryl S-200 was purchased from Pharmacia, Uppsala, Sweden, and Phenylagarose from Sigma. [U-^{3}H]L-Glutamate, supplied by Amersham-Searle Corporation, Arlington Heights, Illinois, was diluted with carrier L-glutamate to give a final specific activity of 2.5 µCi/1.5 umole. Dr. B. Schircks Laboratories in Jona, Switzerland supplied pteroyldiglutamic acid and polyglutamate markers. [6 R,S]tetrahydrofolate polyglutamates ($H_4PteGlu_n$, where n = 1-6) were generated by catalytic hydrogenation of the corresponding $PteGlu_n$ derivatives (Reid and Friedkin, 1973). Bray's solution (Bray, 1960), used in all of the liquid scintillation counting, was prepared from scintillation grade chemicals supplied by Sigma Chemical Co. and Fisher Scientific.

2.2 Plant Material

Pea (Pisum sativum L. cv Homesteader) seeds were purchased from Robertson-Pike and Apache Seeds, Edmonton, Canada. The seeds were surface-sterilized by soaking in 1% (v/v) sodium hypochlorite for 5 minutes, rinsed thoroughly with sterile distilled water and then allowed to imbibe sterile distilled water for 18 hours at room temp-

erature. Damaged or non-imbibed seeds were discarded prior to planting or extraction. Germination was carried out in sterile horticultural-grade vermiculite at 25°C in darkness.

For studies using inhibitors, the seeds were imbibed in the inhibitor solution (cycloheximide, 100 µg/ml; chloramphenicol, 100 µg/ml; or methotrexate, 0.1 mM) for 18 hours at room temperature. The treated seeds were then germinated in sterile vermiculite at 25°C in darkness. The flats were watered with sterile distilled water during the subsequent period of growth.

In other experiments, seeds of wheat (Triticum aestivum cv Neepawa), pea (Pisum sativum) and barley (Hordeum vulgare) were imbibed and germinated as described above but were grown either under greenhouse conditions at 25°C or in the dark at room temperature. Seedlings were grown for about 2 weeks and were watered with half-strength Hoagland's solution prior to harvest of plant tissues.

2.3 Assay of Folylpolyglutamate Synthetase (FPGS) Activity

2.3.1 Preparation of folate substrate:

[R,S]tetrahydrofolic acid was prepared from PteGlu by bubbling hydrogen gas through a 0.1 mM solution in the presence of platinum oxide (Reid and Friedkin, 1973). Conversion of folic acid to THFA was checked by measuring the increase in absorbance of the solution. Folic acid has maximal absorption at 280 nm while THFA absorbs strongly at 296 nm (Blakley, 1969). When conversion was completed, the platinum oxide was removed by centrifugation, and 2-mercapto-

ethanol was added to the supernatant to give a final concentration of 1.0 M. The solution was stored at -20°C until required.

2.3.2 Measurement of FPGS activity:

The assay of folylpolyglutamate synthetase was based on the incorporation of [^{3}H]glutamate into folylpolyglutamates as summarized in Table 2. The standard reaction system (in a final volume of 1 ml) contained: Tris-HCl pH 8.5, 100 µmol; $MgCl_2$, 5 µmol; ATP, 5.0 µmol; [R,S]-H_4PteGlu, 0.1 µmol; 2-mercaptoethanol, 100 µmol; L-glutamate, 1.5 µmol containing 2.5 µCi of [^{3}H]glutamate; and extract protein. The reaction tubes were purged with hydrogen and incubated at 37°C for 2 hours. The reaction was terminated by addition of 3 ml of 93 mM sodium acetate pH 5.2 (Pristupa et al., 1984) followed by incubation at 100°C for 5 minutes. The tubes were then stored at 4°C prior to isolation of the labelled products.

Ion-exchange chromatography was used to separate [^{3}H]glutamate from the [^{3}H]folylpolyglutamate products (Pristupa et al., 1984). Cellex D cellulose was washed with water, decanted several times to remove the fines and equilibrated with 0.07 M sodium acetate (pH 5.2). Bio-Rad Econocolumns (1 x 3 cm) were packed with the equilibrated Cellex D and a 0.5 cm layer of acid-washed sand suspended in 0.07 M sodium acetate (pH 5.2) was placed on top of the bed to prevent disturbance of the anion exchange matrix.

Table 2. Major steps in the assay of folylpolyglutamate synthetase activity.

Step	Reaction and Isolation Procedures
1.	Incubation of reaction system at 37°C*
2.	Termination of reaction by addition of Sodium acetate (pH 5.2) and boiling for 5 min.
3.	Removal of denatured protein by centrifugation.
4.	Supernatant applied to 3 x 1 cm columns of DEAE-cellulose.
5.	^{3}H-Glutamate removed by washing columns with 40 ml Na acetate (pH 5.2).
6.	Labelled polyglutamates recovered by washing column with 6 ml 0.2 N HCl.
7.	1 ml aliquots of acid fraction for scintillation counting (dioxane-based fluor).

* The standard reaction system contained: Tris-HCl pH 8.5, 100 umol; $MgCl_2$, 5 µmol; ATP, 5.0 µmol; [R,S]H_4PteGlu, 0.1 µmol; 2-mercaptoethanol, 100 µmol; L-glutamate, 1.5 µmol containing 2.5 µCi of [^{3}H]glutamate; and extract protein.

Acidified and boiled reaction mixtures were centrifuged in a table top centrifuge to remove precipitated protein. The supernatants were then applied to the DEAE-cellulose columns. The assay tubes were rinsed with 1 ml of 0.07 M sodium acetate (pH 5.2) and this rinse was added to the columns after the sample had penetrated the beds. Following this, each column was washed with 40 ml of 0.07 M sodium acetate pH 5.2 (Pristupa et al., 1984) to remove unreacted [^{3}H]glutamate. [^{3}H]polyglutamates were then eluted by adding 5 ml of 0.2 N HCl to the column after the sodium acetate wash was completed. 1 ml aliquots of the HCl wash were counted in 4 ml of Bray's solution (Bray, 1960).

Control assays lacking THFA were run for each sample to allow correction for any non-folate dependent incorporation of ^{3}H into the polyglutamate fraction.

2.4 Isolation and Purification of Folylpolyglutamate Synthetase

2.4.1 Preparation of cell-free extracts:

Pea seeds were imbibed and germinated as described above (Section 2.2). Seedlings were harvested after periods of 18 hours, 2, 3, or 4 days. The testas and embryos were removed. Cotyledons were soaked for 1 minute in 70% ethanol, drained, and then soaked again for 1 minute in 1% H_2O_2 to remove bacteria (Coffin, 1985). They were then allowed to stand briefly in ice-cold water prior to homogenization. All steps in the extraction were carried out at 2^{o}C. Samples of the cotyledons (20 to 500 grams fresh weight) were homogenized in 25 to 500 ml of extraction buffer (20 mM potassium

phosphate buffer, pH 7.4 containing 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM benzamidine). The homogenate was filtered through four layers of cheesecloth prior to centrifugation at 30,000 x g for 15 minutes (Chan et al., 1986). Aliquots of the supernatant (Step 1 protein) were then dialyzed overnight against the extraction buffer prior to assay of enzyme activity.

2.4.2 Purification of FPGS:

All steps in the purification procedure were performed at 2°C. Streptomycin sulphate (10% w/v; dissolved in extraction buffer, pH 7.4) was added to the crude extract (Step 1 protein) to give a final concentration of 1% (Cichowicz and Shane, 1987). After stirring for 1 hour, the precipitate was removed by centrifugation at 30,000 x g for 15 minutes using a Beckman JA-20 rotor. The resulting supernatant was designated Step 2 protein.

Solid ammonium sulphate was then slowly added to the supernatant (Chan et al., 1986). Protein precipitating between 35 to 45% of saturation was collected by centrifugation at 30,000 x g for 15 minutes. The resulting pellets were redissolved in a small volume (5-25 ml) of extraction buffer to give Step 3 protein. This extract was subjected to overnight dialysis against 500 volumes of the extraction buffer prior to enzyme assay. If further purification was undertaken, these extracts were dialyzed for only 1 hour against extraction buffer before gel filtration. In this case, 10 to 15 ml of extract was applied to 2.6 x 80 cm columns of Sephacryl S-200.

The extraction buffer was pumped through the column at a flow rate of 0.5 ml per minute. Fractions of 6 ml were collected. FPGS activity was located in fractions 37 to 43. These were pooled to give Step 4 protein.

The pooled sample (Step 4 protein, 102 mls) was next applied to a 2.6 x 52 cm column of DE-52 cellulose which had previously been equilibrated with 30 mM potassium phosphate buffer pH 7.0 containing 10 mM KCl, 50 mM 2-mercaptoethanol, 1 mM PMSF and 2 mM benzamidine. The column was washed with an equivalent volume of this buffer. FPGS activity was eluted by applying a linear KCl gradient (300 ml of buffer containing 10 mM KCl in the mixing vessel to 300 ml of the same buffer in the reservoir but containing 600 mM KCl). Fractions of 6 ml were collected. Fractions containing FPGS activity (fractions 79 to 83) were pooled (Step 5 protein) and dialyzed for 1 hour against the extraction buffer to remove the K^+ and Cl^- ions prior to chromatography on Phenylagarose.

A saturated ammonium sulphate solution (prepared by adding saturating amounts of ammonium sulphate to the extraction buffer) was added to the Step 5 protein to give 60% of saturation with $(NH_4)_2SO_4$. The solution was centrifuged and the resulting pellet was redissolved in a small volume of buffer containing 10% ammonium sulphate. This extract (4.5 ml) was then applied to a 1 x 10 cm column of Phenylagarose which had previously been equilibrated with extraction buffer containing 10% ammonium sulphate (Cichowicz and Shane, 1987). After application of the sample, the column was washed with 30 mls of this same buffer. The column was then eluted

with a decreasing linear gradient of ammonium sulphate in the extraction buffer (125 ml of buffer containing 10% ammonium sulphate in the mixing vessel to 125 ml of buffer lacking ammonium sulphate in the reservoir). After the gradient elution had been completed, the column was washed with extraction buffer alone to elute FPGS activity. Fractions of 6 ml were collected and those containing FPGS activity (fractions 39 to 56) were pooled to give Step 6 protein.

2.5 Properties of Folylpolyglutamate Synthetase

In most cases, step 5 protein was used to determine the properties of the pea cotyledon FPGS. The effect of ATP concentration on the rate of the FPGS reaction was determined using amounts ranging from 0 to 5 µmoles/assay. The effect of THFA and L-glutamate concentration on the reaction rate was determined using amounts ranging from 0 to 100 nmoles/assay and 0 to 2.5 µmoles/assay, respectively. Michaelis-Menton kinetics were determined for each using Lineweaver-Burk plots as described in Dixon and Webb (1964).

2.6 Identification of Folate Polyglutamates

2.6.1 Folate cleavage and HPLC analysis:

Folates from pea cotyledon extracts were cleaved to p-aminobenzoate polyglutamate ($pABAGlu_n$) derivatives following the method outlined by Shane, 1986. In this method, 2 ml extracts were adjusted to pH 1 with 5 N HCl and left at 4°C overnight to convert 5- and 10-formyl-$H_4PteGlu_n$ to 5,10-methenyl-$H_4PteGlu_n$. The solutions were adjusted to pH 6.0 with 5 N NaOH and $NaBH_4$ (100 mg) and n-octanol (1 drop) were immediately added. The borohydride

treatment, which lasted 15 minutes, converted 5,10-methenyl-H_4PteGlu$_n$ to 5-methyl-H_4PteGlu$_n$. The excess $NaBH_4$ was destroyed by acidification. Approximately 1 ml of 0.2 M $HgCl_2$ was then added to precipitate any 2-mercaptoethanol present in the extract. The white precipitate formed in this step was removed by centrifugation and the resulting solutions were stored with occasional shaking for 4 hours at room temperature after adjusting the pH to 12. This step allowed the oxidation of 5-methyl-H_4PteGlu$_n$ to 5-methyl-H_2PteGlu$_n$ and the deformylation of 10-formyl-PteGlu$_n$. The solutions were adjusted to pH 1 and stored overnight at 4°C, allowing for the cleavage of 5-methyl-H_2PteGlu$_n$ to pABAGlu$_n$. As well, 5,10-methylene-H_4PteGlu$_n$, H_4PteGlu$_n$ and H_2PteGlu$_n$ are all quantitatively cleaved to pABAGlu$_n$ by this treatment. 5 N HCl (0.1 vol) and Zn dust (0.05 volume of a suspension containing 1 g Zn in 4 ml 0.5% w/v gelatin) were next added to the solutions. The mixtures were shaken intermittently for 5 minutes to cleave PteGlu$_n$ to pABAGlu$_n$. The mixtures were filtered to remove the Zn.

The H_4PteGlu$_n$ formed in FPGS assays was also cleaved to pABAGlu$_n$ and analyzed by HPLC (Shane, 1986). After removal from small (1 x 3 cm) DEAE-cellulose columns, 4 or 5 ml solutions containing H_4PteGlu$_n$ in 0.2 N HCl were adjusted to pH 1 with 5 N HCl and stored overnight at 4°C. The folates were then cleaved to pABAGlu$_n$ using the Zn/acid treatment described above.

pABAGlu$_n$ was purified by diazotization and conversion to the azo dye of naphthylethylene diamine (Brody et al., 1979). The solutions were applied to 0.7 x 4 cm columns of BioGel P2

equilibrated with 0.1 N HCl. The columns were washed with 0.1 N HCl (6 x 1 ml) to remove the pterin derivative and nonspecific ultraviolet absorbing material. The azo dye derivatives were eluted by applying 1 ml of 0.1 M sodium phosphate buffer (pH 7) followed by water (5 x 1 ml). The eluents were adjusted to pH 1. Zn dust (0.05 volume of a suspension containing 1 g Zn in 50 ml 0.5% w/v gelatin) was added and the mixtures were shaken periodically for 15 minutes to allow the reconversion to $pABAGlu_{11}$ to occur. Excess Zn was removed by filtration and the solutions were concentrated to dryness at $50^{0}C$ under reduced pressure. The resulting residues were redissolved in approximately 0.5 to 1.3 ml of H_2O and the pH was adjusted to 6.5. After removal of zinc hydroxide by centrifugation, the extracts were clarified by filtration through 0.45 um BioRad filters.

Folate derivatives were then separated by HPLC using a Varian 5000 chromatography system (Shane, 1986). A silicon precolumn was connected before the injection port. A 2.1 x 70 mm (ID) survival column containing a strong anionic exchanger (Whatman) was placed between the injection port and the analytical column. Aliquots (usually 100 ul) of $pABAGlu_n$ were separated on a 4.6 x 250 mm (ID) microparticular strong anionic exchanger column (Partisil 10 SAX, Whatman). The flow rate was set to 1 ml/minute and the column temperature was $35^{0}C$. The column was eluted isocratically for 10 minutes with 25 mM ammonium phosphate buffer, pH 6.5. Following this, a buffer gradient of ammonium phosphate from 25 mM to 275 mM was run for 30 minutes. A gradient running from 275 mM to 500 mM over 45 minutes followed. The column was reequilibrated by changing

the buffer concentration from 500 mM to 25 mM over a 5 minute period followed by a 15 minute wash using 25 mM ammonium phosphate. One ml fractions were collected and measured by scintillation counting for [^{3}H]pABAGlu$_n$ samples. Polyglutamate samples, derived from pea cotyledon extracts, were assayed for pABAGlu$_n$ by measuring absorbance at 280 nm. Peak area counts were then used to determine the distribution of the naturally occurring polyglutamate derivatives.

2.7 Measurement of Radioactivity by Liquid Scintillation

Radioactive samples were counted using a Tracor Analytic Liquid Scintillation counting system (Mark III, Model 6881). Disintegrations per minute (dpm) were calculated from quench correction curves prepared using radioactive standards. Bray's solution (Bray, 1960) was used for all of the scintillation counting performed.

2.8 Protein Determination

Protein was determined using the method of Bradford (1976) as 2-mercaptoethanol does not interfere with this assay. Bovine serum albumin (Calbiochem, Fraction V) was used in the standard curves that were prepared for each assay.

III. RESULTS

3.1 Folylpolyglutamate Synthetase Assay

The assay of folylpolyglutamate synthetase activity used in this study involved the incorporation of [^{3}H]-labelled glutamate into polyglutamate products. Unincorporated [^{3}H]glutamate was then separated from labelled polyglutamate by chromatography on small columns of DEAE-cellulose, as described in the Materials and Methods. The elution conditions separated [^{3}H]glutamate from the [^{3}H]folylpolyglutamates, as shown in Figure 3.

The results presented in Table 3 confirm that product formation, like that of other FPGS assays (Pristupa et al., 1984; Shane, 1980; Cichowicz and Shane, 1987), was dependent on ATP, folate, and enzyme. Although the assay conditions and substrates were appropriate and optimal for folylpolyglutamate synthetases from bacterial, fungal, and mammalian sources, confirmation was needed for the synthetase from pea cotyledons. The data in Table 3 show that the complete reaction system incorporated 11.2 nmoles of L-glutamate into polyglutamates over a 2 hour period. Removal of tetrahydrofolic acid, ATP, L-glutamate, or enzyme dropped polyglutamate synthesis to minimal levels. The enzyme showed a strict requirement for the reducing conditions supplied by 2-mercaptoethanol. Mg^{2+} was also required, as the substrate form of ATP used by the enzyme was MgATP. As well, incorporation of [^{3}H]glutamate into folylpolyglutamates was close to linear in the 2 hour assay period when various amounts of protein were added. Therefore the assay used in earlier studies of

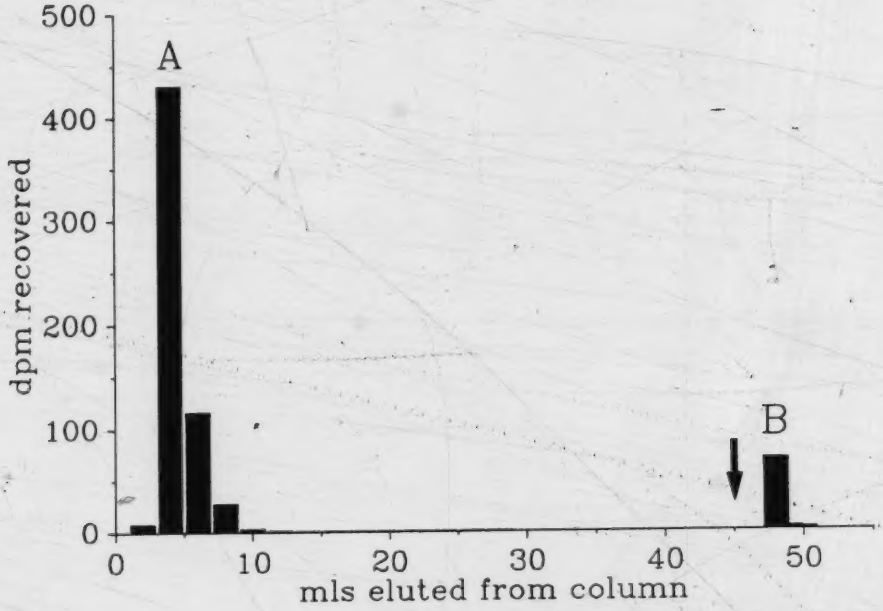


FIGURE 3

Chromatographic separation of [^{3}H]glutamate and [^{3}H]folylpolyglutamates.

Standard reaction mixtures (see Materials and Methods) were applied to 1 x 3 cm columns of DEAE-cellulose equilibrated with 0.07 M sodium acetate pH 5.2. A, elution of unincorporated [^{3}H]glutamate (x 10^{-5}). B, elution of [^{3}H]polyglutamates (x 10^{-3}). The arrow indicates the point at which 5 mls of 0.2 N HCl was added.

Table 3. Requirements for the formation of folylpolyglutamates by folylpolyglutamate synthetase from 18 hour old pea cotyledons.

System	nmoles L-Glutamate Incorporated Into Polyglutamates
Complete*	11.2
-tetrahydrofolate	0.0
-ATP	0.0
-L-glutamate	2.3
-enzyme	0.2

*Standard assay conditions were used (see Materials and Methods) except that single components were omitted. Enzyme activity was obtained from 35-45% ammonium sulphate fractionation (Step 3 protein). Reactions were incubated for 2 hours at 37°C.

bacterial, fungal and mammalian synthetases was applicable in the present study.

3.2 Folylpolyglutamate Synthetase in Plant Tissues

Before attempting to purify and characterize folylpolyglutamate synthetase, it was first necessary to find a suitable plant source of this enzyme. Crude extracts of a variety of plant tissues were assayed for folylpolyglutamate synthetase activity. The results obtained from this study are shown in Table 4. Folylpolyglutamate synthetase activity could not be detected in crude extracts of light-grown and etiolated wheat or barley leaves. In addition, crude extracts of 3 day old pea embryos and pea roots appeared to lack detectable activity under the reaction conditions employed. On the other hand, pea leaf tissue gave activities of 0.24 and 0.48 nmoles [^{3}H]glutamate incorporated per mg protein for crude and ammonium sulphate extracts, respectively. Based on this limited survey, the best source of enzyme activity was determined to be in pea cotyledons and these were chosen as an enzyme source in all further studies.

The amount of folylpolyglutamate synthetase activity present in pea cotyledons was measured during the first four days of germination (Table 5). Crude extracts were examined for FPGS activity after 18 hours of imbibition and after two days and four days of germination. The specific activity of the enzyme did not change markedly over the four day period. In this sense, the enzyme was as active at 18 hours (1.4 ± 0.1 and 1.2 ± 0.1) as it was after four days (1.2 ± 0.2 and 1.2 ± 0.1) of germination.

Table 4. Distribution of folylpolyglutamate synthetase in plant tissues.

Plant Tissue*	FPGS Activity (units/mg protein)**
Wheat leaf (light grown)	n.d.***
Wheat leaf (etiolated)	n.d.
Barley leaf (light grown)	n.d.
Barley leaf (etiolated)	n.d.
Pea cotyledon (18 hour imbibition)	0.37
Pea cotyledon (18 hour imbibition) (35-45% ammonium sulphate extract)	1.53
Pea embryo (3 day)	n.d.
Pea root (15 day)	n.d.
Pea root (15 day) (35-45% ammonium sulphate extract)	n.d.
Pea leaf (15 day)	0.24
Pea leaf (15 day) (35-45% ammonium sulphate extract)	0.48

* All assays for FPGS activity were carried out using crude extracts of plant tissue unless otherwise indicated.

** One unit of FPGS activity is equal to 1 nmole [^{3}H]glutamate incorporated in 1 hour under standard assay conditions.

***[^{3}H] recovered in polyglutamate fraction less than two times that of -THFA control.

Table 5. Specific activity of folylpolyglutamate synthetase from crude extracts of pea cotyledons after 18 Hours, 2 days and 4 days of germination.

Time	Specific Activity (units/mg protein)*
18 hour	1.4 ± 0.1
	1.2 ± 0.1
2 day	0.9 ± 0.1
	1.2 ± 0.2
4 day	1.2 ± 0.2
	1.2 ± 0.1

*One unit of FPGS activity is equivalent to 1000 dpm of radioactivity incorporated in one hour at 37°C.

Pea seeds were imbibed in cycloheximide and chloramphenicol as described in the Materials and Methods section of this thesis. Cycloheximide is known to inhibit cytosolic protein synthesis while chloramphenicol inhibits protein synthesis in organelles (Avers, 1986). The cycloheximide concentration used (100 µg/ml) was sufficient to inhibit germination as radicle and shoot emergence were prevented even after four days of germination. Seeds germinated in water for this same time period had radicles approximately 5 cm in length and shoots approximately 2 cm in length. However, as the data in Table 6 show, the presence of cycloheximide had little or no effect on enzyme activity when compared to water controls, indicating that an active form of cytosolic folylpolyglutamate synthetase is probably present in the seed prior to imbibition and germination. The chloramphenicol concentration used (100 µg/ml) inhibits organelle protein synthesis in pea cotyledons (Coffin, 1985) although visual observations of 4 day old seedlings showed little effect on growth of the radicles when compared to water controls. Chloramphenicol did not appear to affect the maintenance of folylpolyglutamate synthetase activity over the four day period examined (Table 6).

Pea seeds were also imbibed in methotrexate (0.1 mM), a folate analogue, to see if this had an effect on the activity of folyl-polyglutamate synthetase. At this concentration, methotrexate produces folate deficiency in pea cotyledons (Roos and Cossins, 1971). As the data presented in Table 6 show, imbibition in methotrexate had little or no effect on enzyme activity over the four day sampling period. However, from visual observations made prior to

Table 6. The effect of cycloheximide (CHI), chloramphenicol (CAP) and methotrexate (MTX) on folylpolyglutamate synthetase activity.

Sample	Specific Activity (units**/mg protein)	Units/g fr. wt.	Units/cotyledon
CHI			
18 hour	1.5 ± 0.2	37.2 ± 4.5	9.4 ± 1.1
	1.2 ± 0.1	32.5 ± 1.0	8.1 ± 0.3
2 day	1.2 ± 0.1	26.5 ± 1.5	7.2 ± 0.5
	1.1 ± 0.0	58.7 ± 1.1	7.7 ± 0.3
4 day	1.0 ± 0.1	21.0 ± 1.9	5.8 ± 0.5
	1.1 ± 0.2	26.7 ± 4.7	7.2 ± 1.3
H_2O			
18 hour	1.4 ± 0.1	32.1 ± 2.0	8.1 ± 0.5
	1.2 ± 0.1	29.2 ± 3.0	7.6 ± 0.8
2 day	0.9 ± 0.1	21.9 ± 2.7	6.0 ± 0.8
	1.2 ± 0.2	31.5 ± 4.5	8.1 ± 0.5
4 day	1.2 ± 0.2	33.8 ± 4.5	8.8 ± 1.2
	1.2 ± 0.1	33.8 ± 2.4	8.9 ± 0.7
CAP			
18 hour	1.5 ± 0.4	36.9 ±10.9	9.2 ± 2.7
	1.0 ± 0.2	25.8 ± 6.9	6.4 ± 1.7
2 day	1.2 ± 0.4	33.0 ±11.1	8.9 ± 3.0
	1.4 ± 0.2	31.1 ± 4.8	8.3 ± 1.3
4 day	1.5 ± 0.4	36.4 ±10.2	9.4 ± 2.6
	1.6 ± 0.2	36.8 ± 3.8	9.8 ± 1.0
H_2O			
18 hour	1.2 ± 0.2	29.3 ± 5.5	7.4 ± 1.4
	1.2 ± 0.4	32.9 ± 9.0	8.1 ± 2.2
2 day	1.5 ± 0.1	32.8 ± 1.2	8.9 ± 0.4
	1.0 ± 0.1	27.8 ± 2.1	7.6 ± 0.6
4 day	1.5 ± 0.2	40.8 ± 4.8	11.0± 1.3
	1.6 ± 0.2	43.5 ± 5.3	11.7± 1.4
MTX*			
18 hour	1.4	34.0	8.5
	1.5	36.6	9.0
2 day	1.4	38.1	10.6
	1.2	26.0	7.0
4 day	1.7	38.9	10.8
	1.7	34.1	9.2
H_2O*			
18 hour	1.8	34.7	9.0
	1.2	27.5	7.0
2 day	1.1	29.7	8.0
	1.5	38.9	10.6
4 day	1.5	32.7	8.7
	1.2	33.6	9.0

* Data for these samples represent means of duplicate assays only.
**1 unit equals 1000 dpm of radioactivity incorporated into polyglutamate per hour.

extraction, methotrexate did inhibit germination. Radicles had barely emerged from the testa even after four days of germination.

3.3 Purification of Folylpolyglutamate Synthetase

Several workers have reported difficulty in purifying folylpolyglutamate synthetases of bacterial, mammalian, and fungal sources, mainly due to the extreme instability and low yield of this enzyme (for a review, see McGuire and Coward, 1984). These two factors also played a role when the present research was undertaken. Initially, attempts to purify the FPGS activity from pea cotyledons were unsuccessful. A description of some of the problems encountered in attempting to purify the enzyme, together with the steps taken to overcome these problems may be summarized as follows.

The major limiting factor in the purification of the enzyme was its instability. Recovery of enzyme activity could be improved by eliminating or reducing time consuming steps and streamlining the purification protocol in such a way that the whole procedure could be completed in approximately 96 hours. For example, extended dialysis led to dramatic decreases in enzyme activity. However, optimal activity could be achieved when dialysis was limited to only two hours. Thus, dialysis to remove salts prior to ion exchange chromatography was kept to a short period of time.

Storage at 4°C for extended periods of time decreased folylpolyglutamate synthetase activity especially as purification progressed. Studies of enzyme stability using activity obtained from Sephacryl S-200 chromatography showed that the enzyme was relatively

stable for four to five weeks at 4°C (Table 7). Addition of bovine serum albumin enhanced enzyme stability, while the presence of DMSO led to a more rapid decline in activity. Enzyme activity dropped very quickly after DEAE-cellulose and Phenylagarose chromatography. To prevent this loss of activity, glycerol to a final concentration of 50% v/v was added to the collected fractions. This treatment stabilized the enzyme, thereby allowing for further purification and characterization. Millipore ultrafiltration units were used in an attempt to concentrate protein at various stages in the purification procedure. However, this treatment tended to lower total FPGS activity.

Another problem encountered in the study of folylpolyglutamate synthetase was that the enzyme was present in low amounts in vivo. The protocol therefore had to be designed in such a way as to keep the recovery as high as possible through each step in the procedure. Phenylmethylsulfonyl fluoride (PMSF) and benzamidine, which are effective protease inhibitors, were routinely added to the extraction buffer and all subsequent buffers to prevent loss of FPGS activity through proteolytic cleavage. A streptomycin sulphate treatment prior to ammonium sulphate fractionation gave 100% recovery of enzyme activity in the Step 3 protein extract. However, if this streptomycin sulphate step was not included, recovery of the enzyme after ammonium sulphate fractionation was only about 30% to 40%.

A typical purification of folylpolyglutamate synthetase activity from pea cotyledons is summarized in Table 8. The protocol adopted was in general like that described by Shane's group for the

Table 7. Stability of folylpolyglutamate synthetase activity after Sephacryl chromatography.

Storage at 4°C (days)	Additions to collected enzyme		
	None	DMSO+	Albumin*
1	100.0	100.0	100.0
4	109.7	99.8	99.9
11	109.7	—	112.5
21	89.3	—	109.0
28	93.6	51.8	108.7
38	86.0	40.1	99.5
48	69.9	31.2	104.8

Data expressed as a percentage of the activity found on day 1.
* 5 mg of bovine serum albumin were added per ml of enzyme sample.
+ Final concentration 20% v/v.

Table 8. Partial purification of pea cotyledon folylpolyglutamate synthetase activity.

Fractionation Step	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific Activity (units/mg)	Purification (x-fold)	Yield (%)
1. Crude extract	540	8.3	9.2	0.3	1.0	100
2. Streptomycin sulphate	559	16.8	9.8	0.5	1.9	210
3. 35-45% (NH_4SO_4)	38	53.8	15.4	1.0	3.9	46
4. Sephacryl S-200	98	57.8	4.7	3.6	13.7	126
5. DEAE-cellulose	16	58.6	0.87	19.8	74.9	21
6. Phenyl-agarose	102	18.7	0.008	723.5	2734	43

1 unit of FPGS activity is the amount catalyzing the incorporation of 1 nmole [^{3}H]glutamate incorporated in one hour under standard assay conditions.

purification of hog liver folylpolyglutamate synthetase (Cichowicz and Shane, 1987). Crude extracts were initially treated with 1% streptomycin sulphate, which removed nucleic acids and other polyanionic species while slightly increasing purification. Ammonium sulphate fractionation (35-45%) concentrated the extract and led to a 3.9-fold enrichment of specific activity. The extract was then applied to a Sephacryl S-200 gel filtration column. A typical elution profile for this column is illustrated in Figure 4. As this Figure shows, most of the applied protein was eluted from the column prior to the elution of FPGS activity. Pooled fractions in the peak area showed an enhanced specific activity (3.6 units/mg protein) and increased purification 13.7-fold over the crude extract.

FPGS activity was strongly bound to anion exchange cellulose (Figure 5). The protein concentration dropped to less than 1 mg/ml in the peak region, leading to an approximately five-fold increase in specific activity compared to step 4 protein. Pooled peak fractions were then applied to Phenylagarose columns (Figure 6). Proteins were eluted using a reverse ammonium sulphate gradient. The bulk of non-specific protein did not bind to the column under these conditions, which led to a further increase in specific activity (724 units/mg protein). Purification of enzyme activity increased 2700-fold over the crude extract.

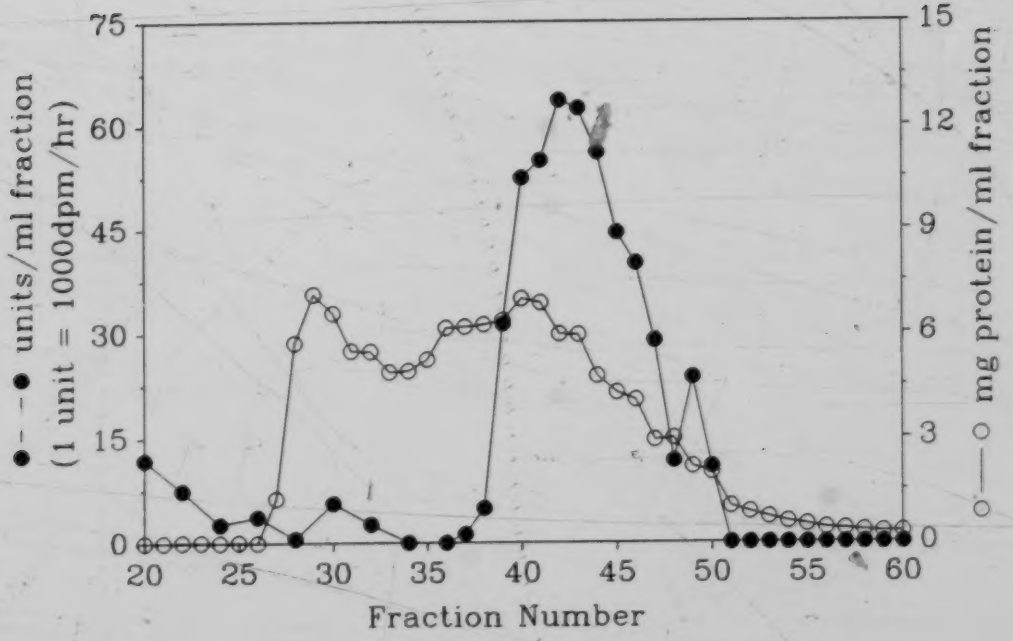


FIGURE 4

SEPHACRYL S-200 CHROMATOGRAPHY OF PEA COTYLEDON FOLYLPOLYGLUTAMATE SYNTHETASE

Step 3 enzyme (12 mls) was applied to a Sephacryl S-200 column (2.6 x 80 cm). The column was washed with 20 mM potassium phosphate buffer pH 7.4 containing 50 mM 2-mercaptoethanol, 2 mM benzamidine and 1 mM PMSF. Fractions of 6 ml were collected and assayed for enzyme activity (●——●) and for protein concentration (o——o).

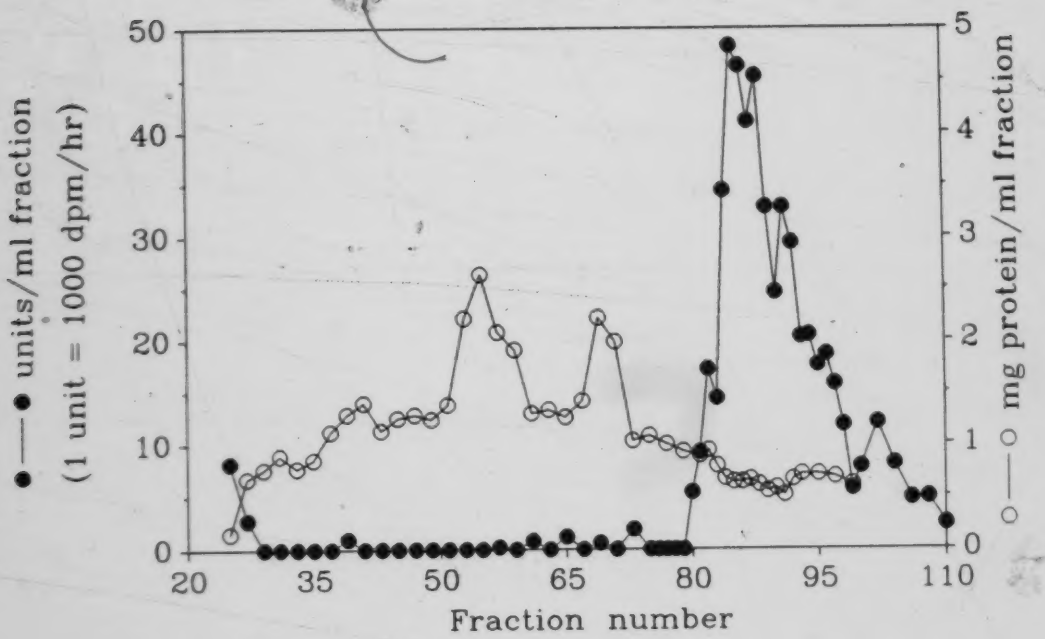


FIGURE 5

ANION EXCHANGE CHROMATOGRAPHY OF PEA COTYLEDON POLYLPOLYGLUTAMATE SYNTHETASE

Step 4 enzyme (102 ml) was applied to a 2.6 x 52 cm column of DEAE-cellulose equilibrated with 30 mM potassium phosphate buffer pH 7.0 containing 10 mM KCl, 50 mM 2-mercaptoethanol, 1 mM PMSF and 2 mM benzamidine. The column was eluted with an increasing gradient of KCl (10 to 600 mM) in buffer. Fractions were examined for enzyme activity (●————●) and for protein concentration (o————o).

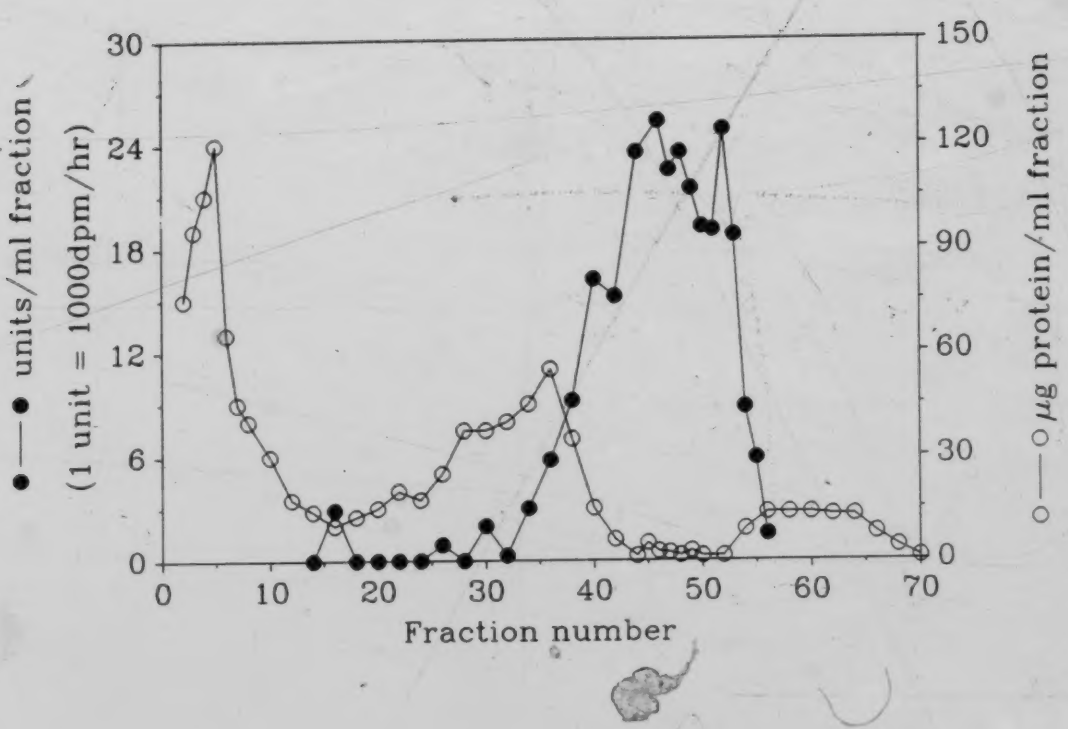


FIGURE 6

PHENYL-AGAROSE CHROMATOGRAPHY OF PEA COTYLEDON FOLYLPOLYGLUTAMATE SYNTHETASE

Step 5 enzyme was applied to a Phenylagarose column (1 x 15 cm) equilibrated with extraction buffer containing 10% ammonium sulphate. The column was washed with 300 mls of the equilibration buffer containing 10% ammonium sulphate, followed by a linear gradient (250 mls) of ammonium sulphate (10% to 0%) in buffer, and finally, with buffer alone (200 mls). Fractions were assayed for enzyme activity (●————●) and protein concentration (o——o).

3.4 Properties of Folylpolyglutamate Synthetase

Folylpolyglutamate synthetase was characterized following its isolation and partial purification from 18 hour old pea cotyledons. In these studies, a number of the enzyme's physical and chemical properties were examined. The results obtained are presented below.

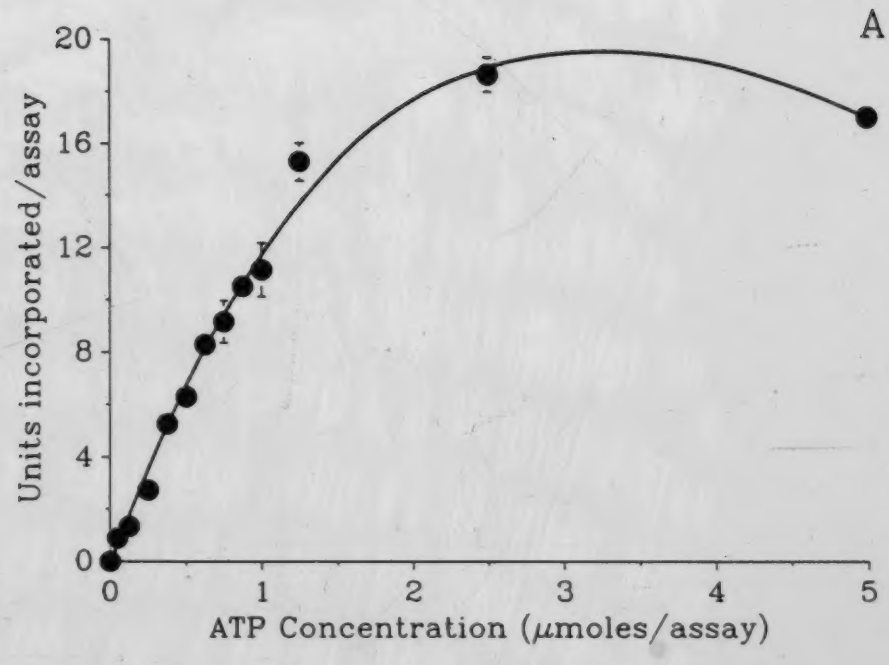
As mentioned in an earlier section, enzyme activity was dependent upon a folate substrate, MgATP, and L-glutamate (Table 3). Under the standard assay conditions (5 mM ATP, 1.5 mM L-glutamate and 100 µM [R,S]H_4PteGlu), MgATP and H_4PteGlu were present in near saturating amounts (Figures 7a and 8a), while L-glutamate was not (Figure 9a). Kinetic plots for these three substrates exhibited typical Lineweaver-Burk kinetics, allowing for the determination of a Michaelis-Menten constant (Km) for each. Figures 7b, 8b and 9b show the Lineweaver-Burk plots used to determine the Km for ATP (2.31 mM), H_4PteGlu (3.51 µM) and L-glutamate (0.61 µM).

Figure 10a illustrates the effect on L-glutamate incorporation as increased amounts of protein were added to the standard assay mixture. In this Figure, increasing the amount of Step 3 protein increased the incorporation of glutamate into polyglutamate. However, similar experiments using Step 5 (DEAE-cellulose) protein showed that the amount of enzyme activity added was saturating at the levels used in the standard assay (Figure 10b). As a result, calculations of specific enzyme activity and therefore of final purification (Table 8) are probably underestimated by two to three fold.

FIGURE 7

EFFECT OF ATP CONCENTRATION ON THE RATE OF THE POLYLPOLYGLUTAMATE SYNTHETASE REACTION.

Reactions were performed under the standard assay conditions using Step 3 protein except that the concentration of ATP was varied as indicated in A. B, Lineweaver-Burk plot of 1/v versus 1/[S]. The Km calculated for ATP from this plot was 2.31 mM.


A
Units incorporated/assay
20
16
12
8
4
0
0
1
2
3
4
5
ATP Concentration (μmoles/assay)

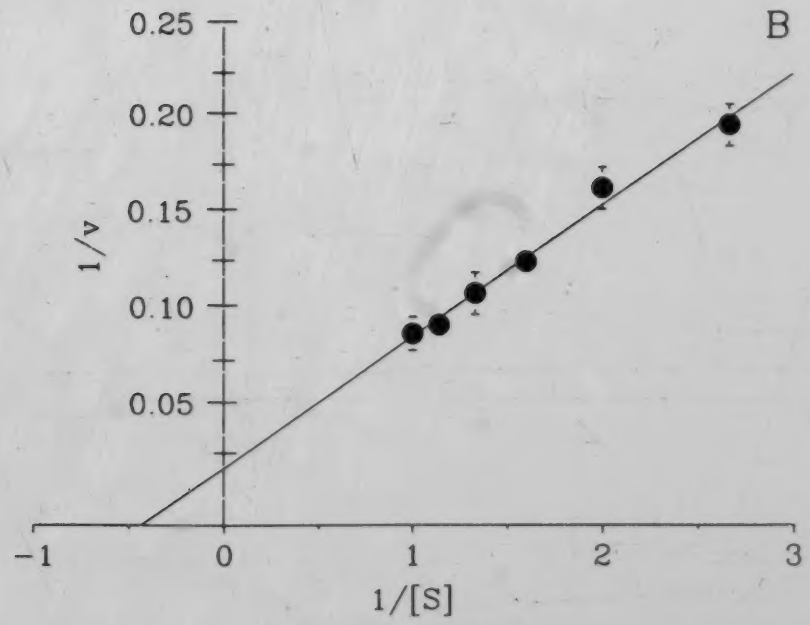
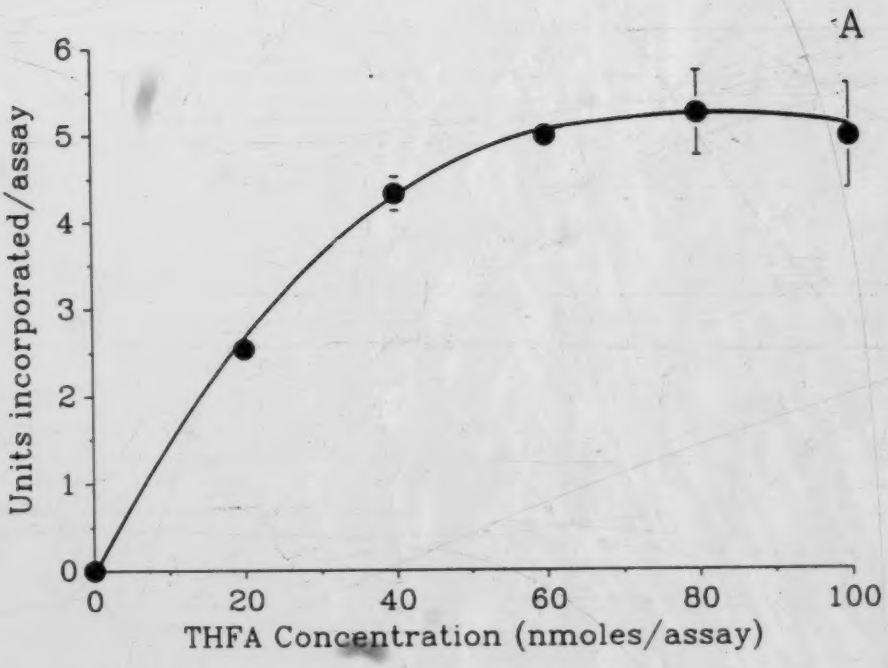

B
0.25
0.20
0.15
0.10
0.05
1/v
−1
0
1
2
3
1/[S]

FIGURE 8

EFFECT OF THFA CONCENTRATION ON THE RATE OF THE POLYLPOLYGLUTAMATE SYNTHETASE REACTION.

Reactions were performed under the standard assay conditions using Step 5 protein except that the concentration of THFA was varied as indicated in A. B, Lineweaver-Burk plot of 1/v versus 1/[S]. The Km calculated for THFA from this plot was 3.51 μM.


A
Units incorporated/assay
0
1
2
3
4
5
6
0
20
40
60
80
100
THFA Concentration (nmoles/assay)

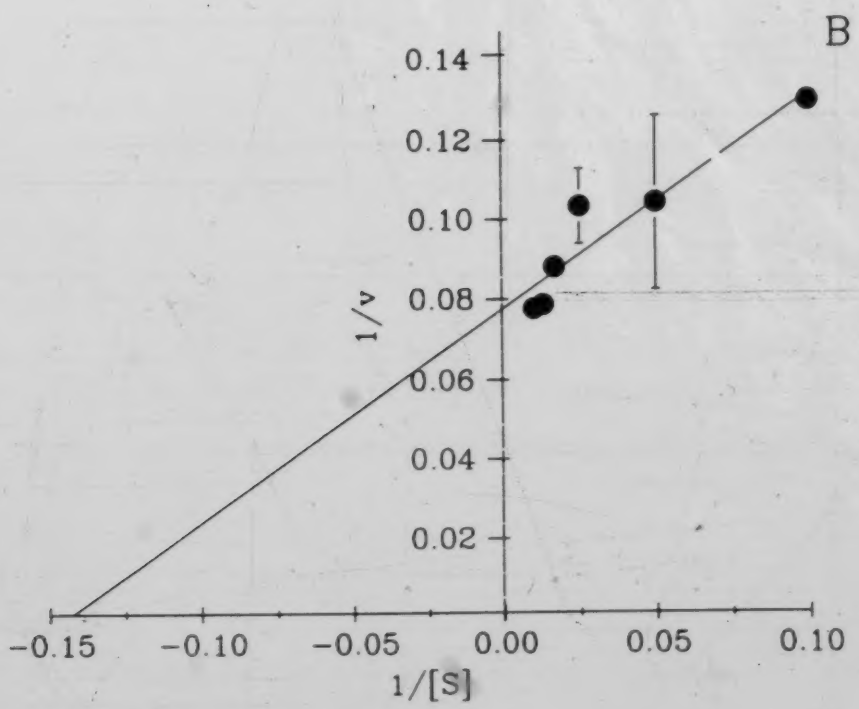
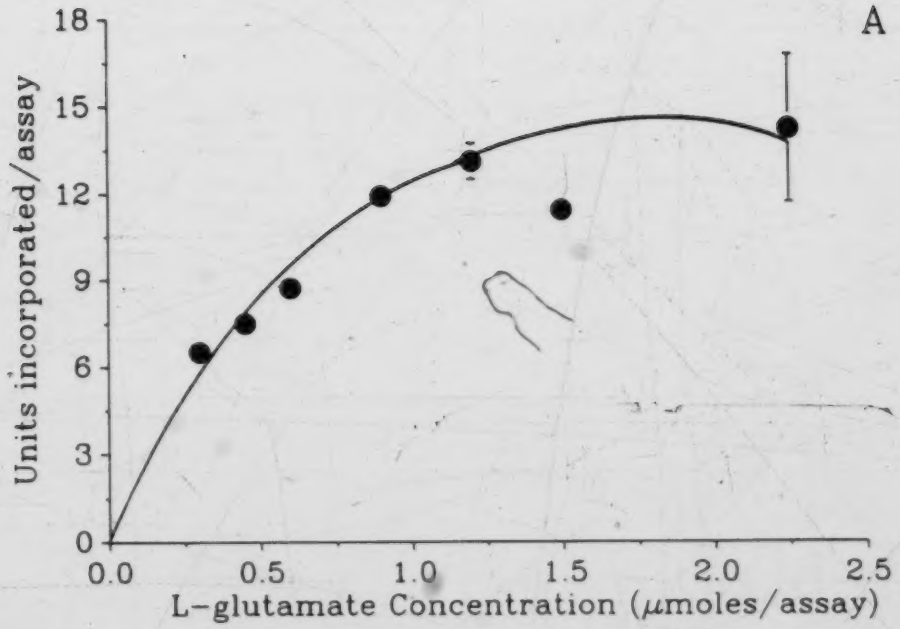

B
1/v
0.14
0.12
0.10
0.08
0.06
0.04
0.02
−0.15
−0.10
−0.05
0.00
0.05
0.10
1/[S]

FIGURE 9

EFFECT OF L-GLUTAMATE CONCENTRATION ON THE RATE OF THE POLYLPOLYGLUTAMATE SYNTHETASE REACTION.

Reactions were performed under the standard assay conditions using Step 5 protein except that the concentration of L-glutamate was varied as indicated in A. B, Lineweaver-Burk plot of 1/v versus 1/[S]. The Km calculated for L-glutamate from this plot was 0.61 μM.


A
Units incorporated/assay
18
15
12
9
6
3
0
0.0
0.5
1.0
1.5
2.0
2.5
L-glutamate Concentration (μmoles/assay)

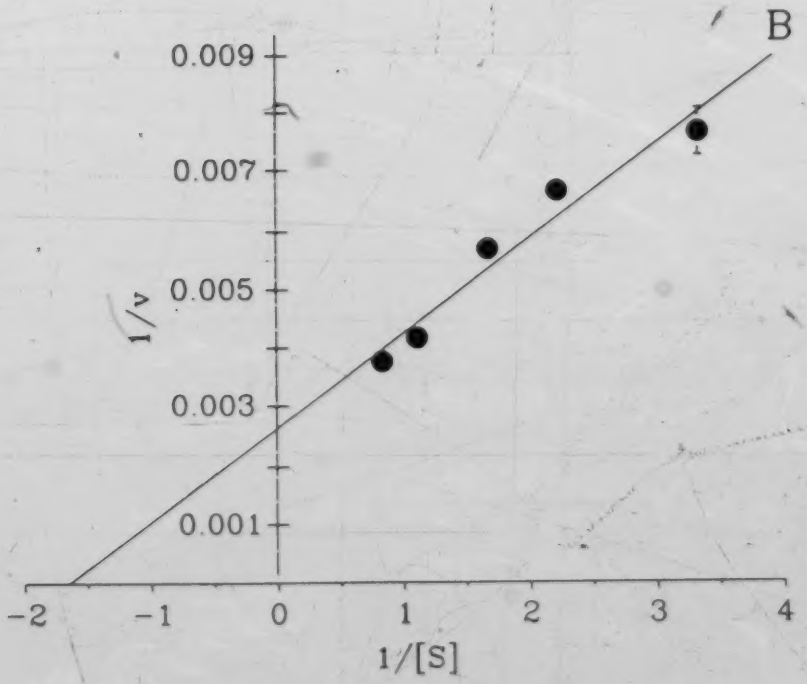
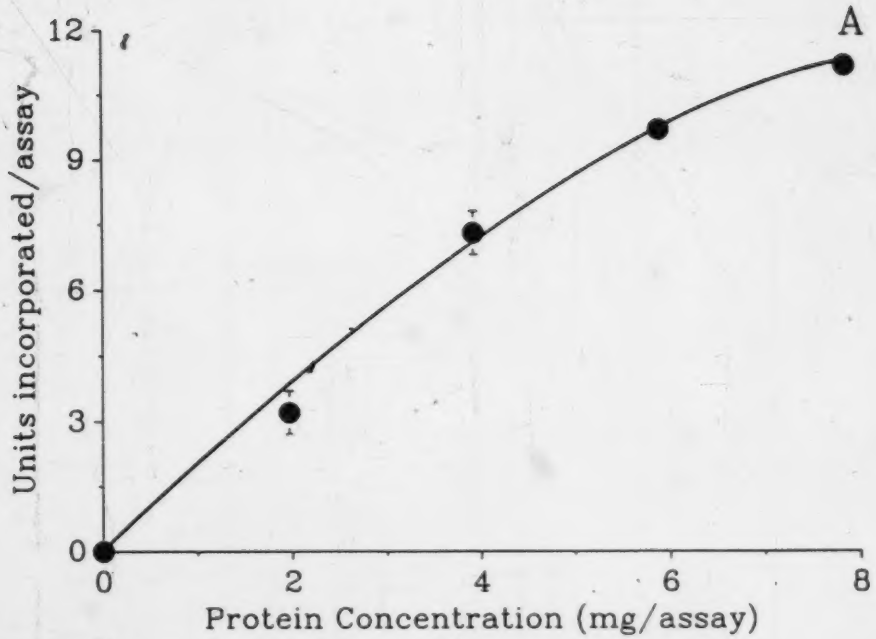
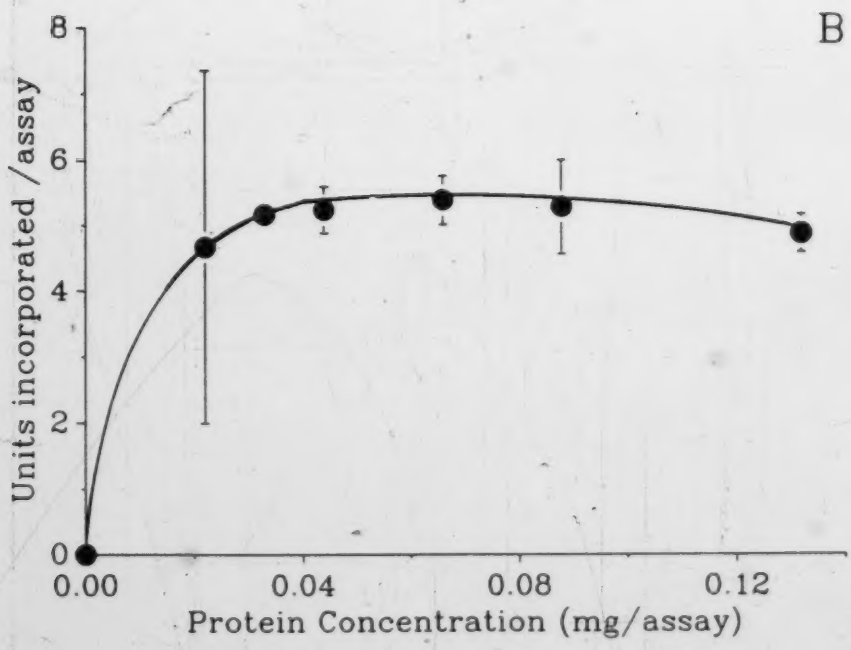

B
0.009
0.007
0.005
0.003
0.001
1/v
-2
-1
0
1
2
3
4
1/[S]

FIGURE 10

EFFECT OF THE ADDITION OF PROTEIN ON THE RATE OF THE POLYLPOLYGLUTAMATE SYNTHETASE REACTION.

Reactions were performed under the standard assay conditions except that the amount of protein added to the assays was varied as indicated. A, effect on incorporation with increasing amounts of Step 3 protein. B, effect on incorporation with increasing amounts of Step 5 protein.


A
12
9
6
3
0
Units incorporated/assay
0
2
4
6
8
Protein Concentration (mg/assay)


B
8
6
4
2
0
Units incorporated /assay
0.00
0.04
0.08
0.12
Protein Concentration (mg/assay)

The pH optimum for folylpolyglutamate synthetase is shown in Figure 11. Tris-HCl buffers ranging from pH 6.0 to 11.0 (22°C) were used to generate the pH range. Enzyme activity increased with increasing pH up to an optimum of 8.5 under standard assay conditions. More alkaline pH values caused a decrease in enzyme activity.

The molecular weight of the enzyme (Figure 12) was estimated using gel filtration (Andrews, 1964). Blue dextran, a high molecular weight compound, was used to determine void volume (Vo). A series of protein molecular weight standards were then eluted and their location in the collected fractions was determined colorimetrically. Ratios of elution volume to void volume (Ve/Vo) were calculated. By plotting the Ve/Vo ratio obtained for folylpolyglutamate synthetase on the line derived from the protein standards, the molecular weight was calculated to be 68,000.

A variety of folate compounds and analogs were tested for their effectiveness as substrates for pea cotyledon folylpolyglutamate synthetase. The results obtained from these tests are presented in Table 9. The greatest amount of activity was observed with 100 uM [R,S]H_4PteGlu. 5,10-Methylene-H_4PteGlu was also an effective substrate. Folic acid, aminopterin, and methotrexate, a folate analogue, were not used as substrates by the enzyme. In other experiments, methotrexate and folic acid were combined with H_4PteGlu to determine if the activity supported by H_4PteGlu would be affected by the presence of these two compounds. It is clear from Table 9 that neither compound had a significant effect on polyglutamate synthesis.

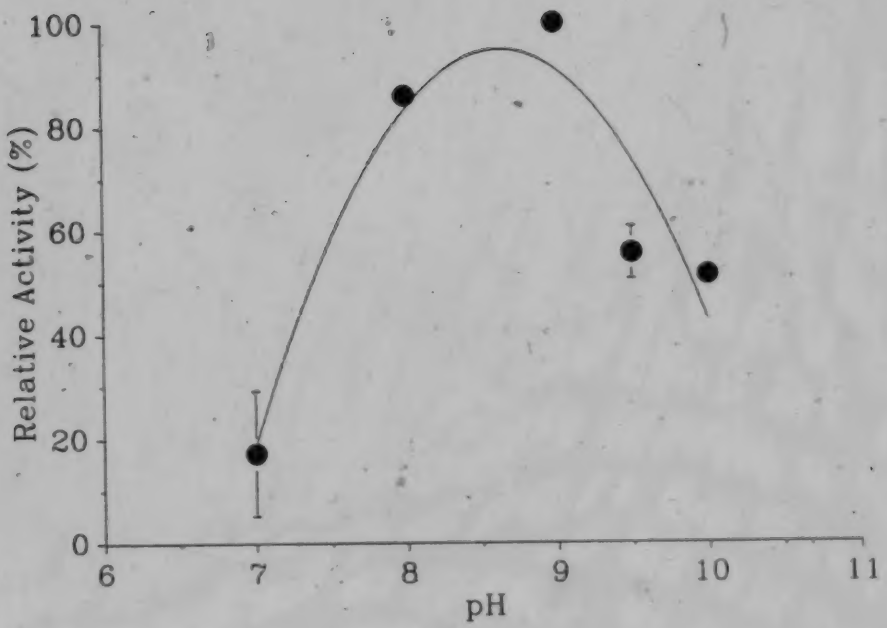


FIGURE 11

THE EFFECT OF pH ON POLYLPOLYGLUTAMATE SYNTHETASE ACTIVITY.

The complete assay system, containing Step 5 protein, was as described in the Materials and Methods except that the buffer used was 100 µM Tris-HCl adjusted to the pH values indicated above with either KOH or HCl.

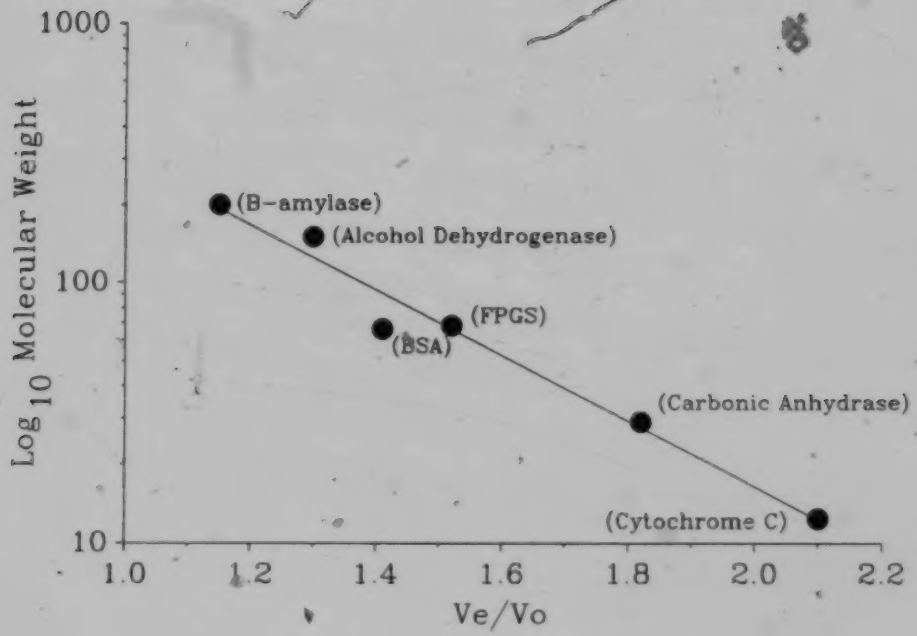


FIGURE 12

DETERMINATION OF THE MOLECULAR WEIGHT OF PEA COTYLEDON POLYLPOLYGLUTAMATE SYNTHETASE.

Molecular weight standards were applied to Sephacryl S-200 gel filtration columns. The volume of buffer required to elute each protein (Ve) was determined by reading the absorbance at 280 nm of each fraction, or, in the case of FPGS, by performing standard assays for activity. The void volume (Vo) of the column was determined by observing the buffer volume required to elute blue dextran.

Table 9. Folate compounds as substrates for folylpolyglutamate synthetase.

Substrate	Substrate Concentration	Relative Activity*
1. [R,S]H_4PteGlu	100 µM	100%
2. PteGlu	100 µM	3.4%
3. [R,S]5,10-Methylene-H_4PteGlu	100 µM	99%
4. [R,S]Methotrexate	100 µM	3.4%
5. Aminopterin	50 µM	3.0%
6. [R,S]H_4PteGlu + [R,S]Methotrexate	100 µM + 100 µM	95.5%
7. [R,S]H_4PteGlu + PteGlu	100 µM + 100 µM	92.9%

Activities are expressed relative to those obtained with [R,S]H_4PteGlu. For 2 and 4, activity obtained with [R,S]H_4PteGlu was 5.2 nmoles L-glutamate incorporated per hour. For 3, 6, and 7, activity obtained with [R,S]H_4PteGlu was 5.8 nmoles L-glutamate incorporated per hour. For 5, activity obtained with [R,S]H_4PteGlu was 4.0 nmoles L-glutamate incorporated per hour.

3.5 HPLC Analysis of [^{3}H]Folylpolyglutamate Products

The products of the reaction catalyzed by folylpolyglutamate synthetase were analyzed for chain length using high performance liquid chromatography following their conversion to $pABAGlu_n$ derivatives (Shane, 1986). The gradient elution parameters used clearly separated polyglutamate derivatives from one another, as Figure 13 illustrates. To obtain this profile, a mixture of polyglutamate standards was applied to a Partisil 10 SAX column as described in the Materials and Methods. Elution of the polyglutamate standards led to a clear separation and the retention time of each derivative was quite specific, repeatable, and in agreement with published data (Shane, 1982).

As the mammalian folylpolyglutamate synthetase is affected by the folate substrate, folate concentration, and time, the present study examined the <u>in vitro</u> chain lengths produced by the pea cotyledon synthetase under a variety of experimental conditions. A number of assays were carried out using Step 5 protein. In these assays, various substrates ($H_4PteGlu$ and $H_4PteGlu_2$) were incubated for two, six or 24 hours at 37^{o}C prior to termination of the reaction. As well, a number of different concentrations of $H_4PteGlu$ (10 µM, 50 µM, 100 µM) were added to determine the effect of the amount of folate substrate on subsequent chain length synthesis. The results of these analyses are presented in Figures 14 to 21. The data are also summarized in Table 10.

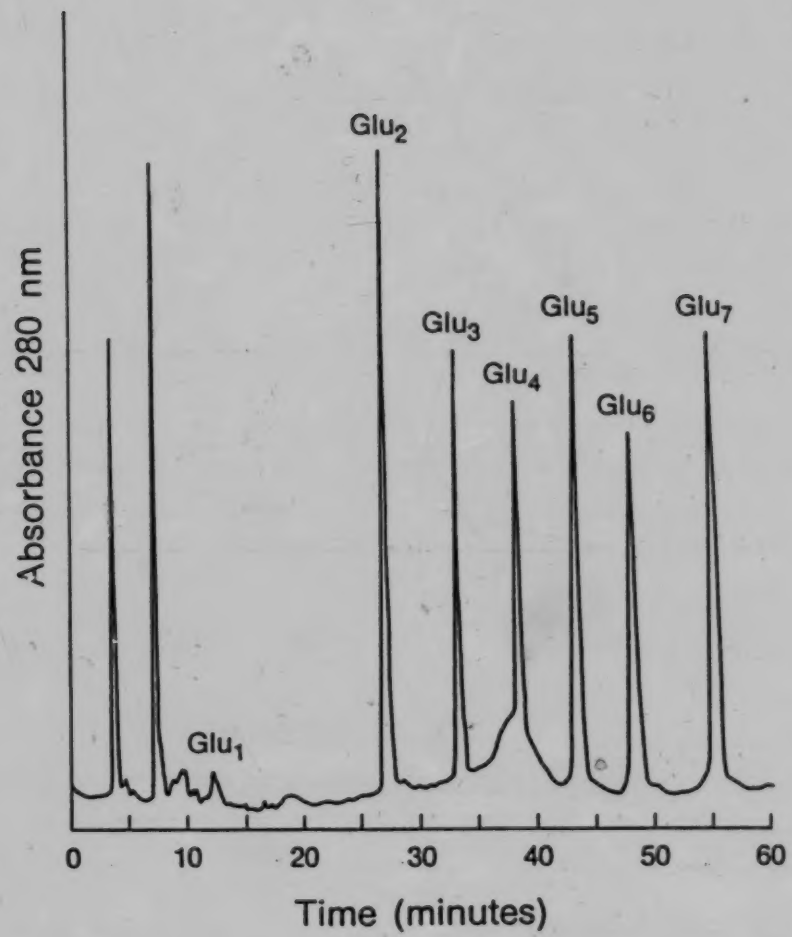


FIGURE 13

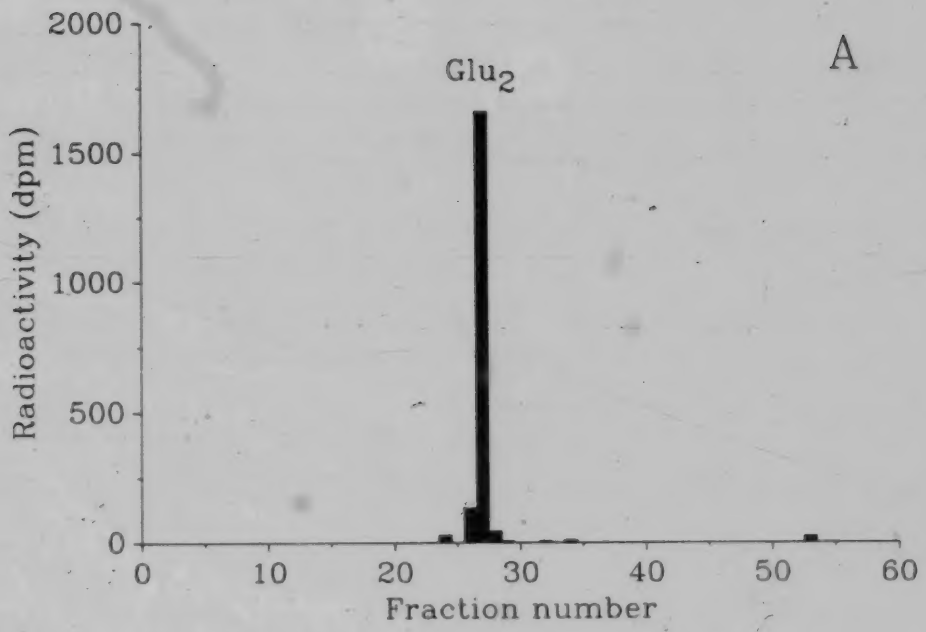
HPLC ELUTION PROFILE OF LABELLED pABAGlu$_{1-6}$ STANDARDS.

Standards were prepared from folate polyglutamate standards and chromatographed on a strong anion exchange column at a flow rate of 1 ml/ min as described in the Materials and Methods.

FIGURE 14

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 2 HOUR FPGS ASSAYS USING 100 μM [R,S]H_4PteGlu AS THE SUBSTRATE.

Standard reaction mixtures containing Step 5 protein and 100 μM [R,S]-H_4PteGlu were assayed in triplicate for 2 hours. The resulting HCl washes containing folylpolyglutamates were pooled and converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent duplicate analyses of the pooled HCl wash.


A
Glu2
2000
1500
1000
500
0
Radioactivity (dpm)
0
10
20
30
40
50
60
Fraction number

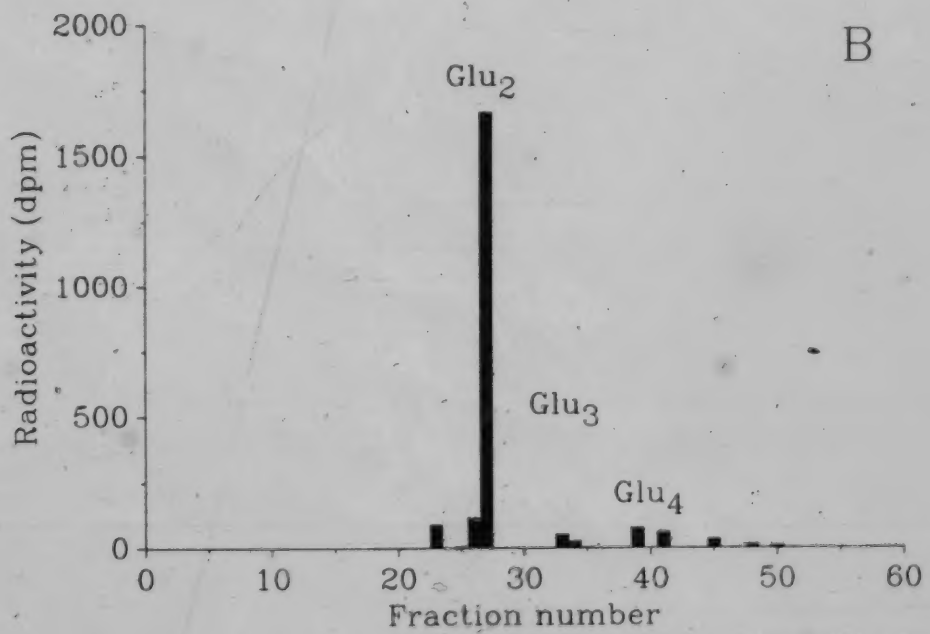
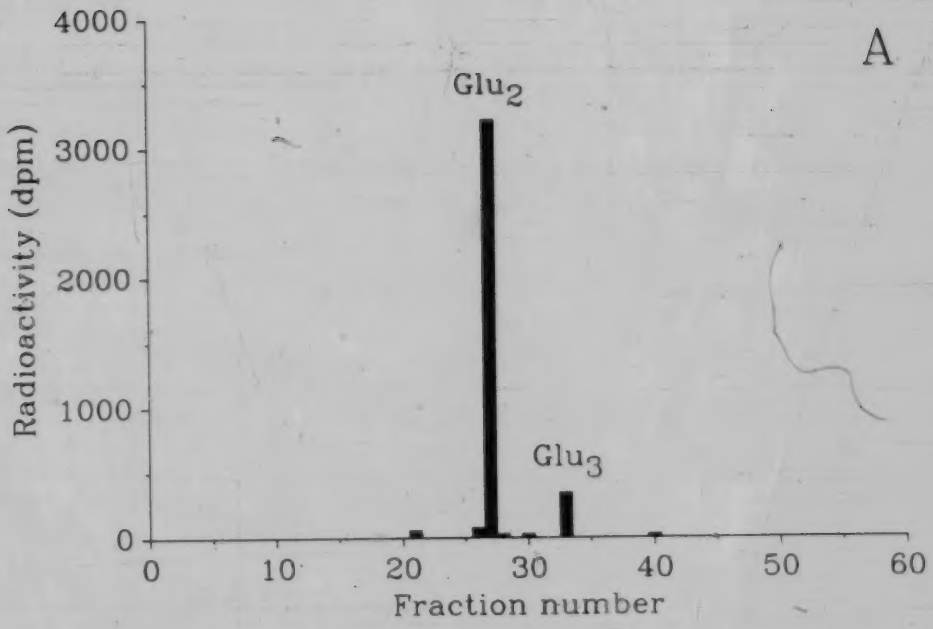

B
Glu2
Glu3
Glu4
2000
1500
1000
500
0
Radioactivity (dpm)
0
10
20
30
40
50
60
Fraction number

FIGURE 15

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 6 HOUR FPGS ASSAYS USING 100 µM $[R,S]H_4PteGlu$ AS THE SUBSTRATE.

Standard reaction mixtures containing Step 5 protein and 100 µM $[R,S]H_4PteGlu$ were assayed in triplicate for 6 hours. The resulting HCl washes containing folylpolyglutamates were pooled and converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fraction of 1 ml were collected and analyzed for radioactivity. A and B represent duplicate analyses of the pooled HCl wash.


A
4000
3000
2000
1000
0
Radioactivity (dpm)
Glu2
Glu3
0
10
20
30
40
50
60
Fraction number

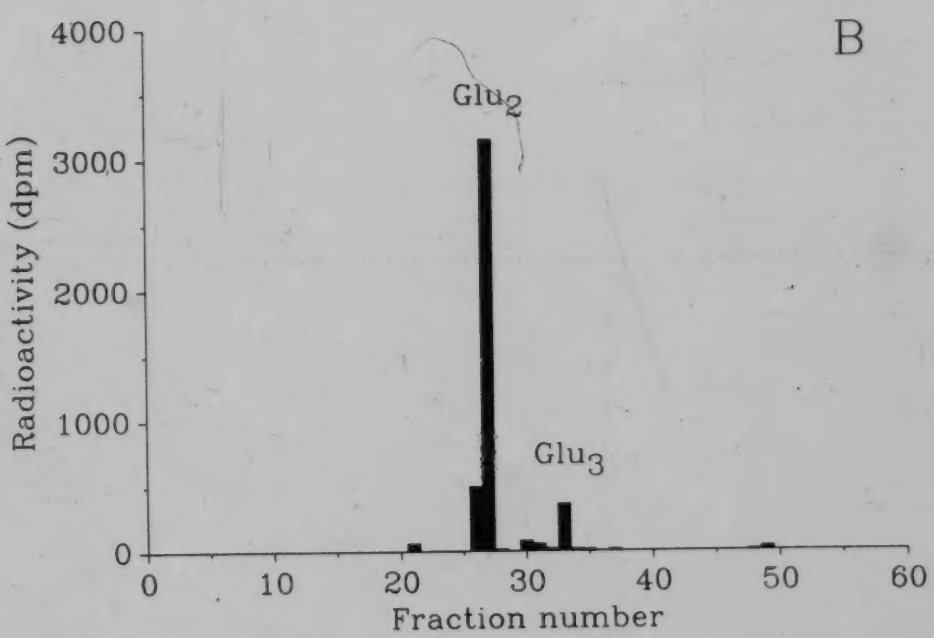
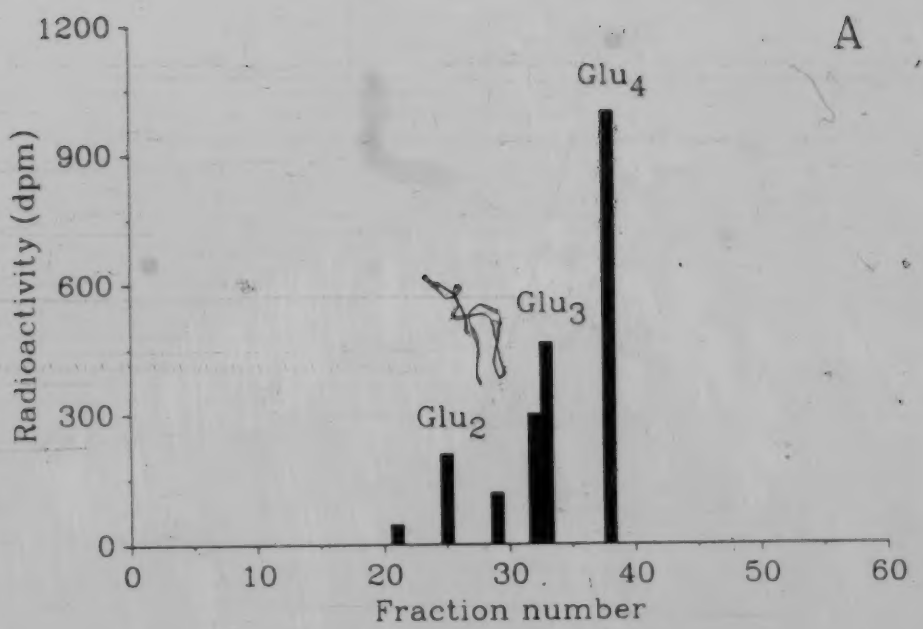

B
4000
3000
2000
1000
0
Radioactivity (dpm)
Glu2
Glu3
0
10
20
30
40
50
60
Fraction number

FIGURE 16

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 24 HOUR FPGS ASSAYS USING 10 µM $[R,S]H_4PteGlu$ AS THE SUBSTRATE.

Reaction mixtures containing Step 5 protein and 10 µM $[R,S]H_4PteGlu$ were assayed in triplicate for 24 hours. The resulting HCL washes containing folylpolyglutamates were converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent products formed from duplicate assays of FPGS activity.


A
1200
900
600
300
0
Radioactivity (dpm)
Glu4
Glu3
Glu2
0
10
20
30
40
50
60
Fraction number

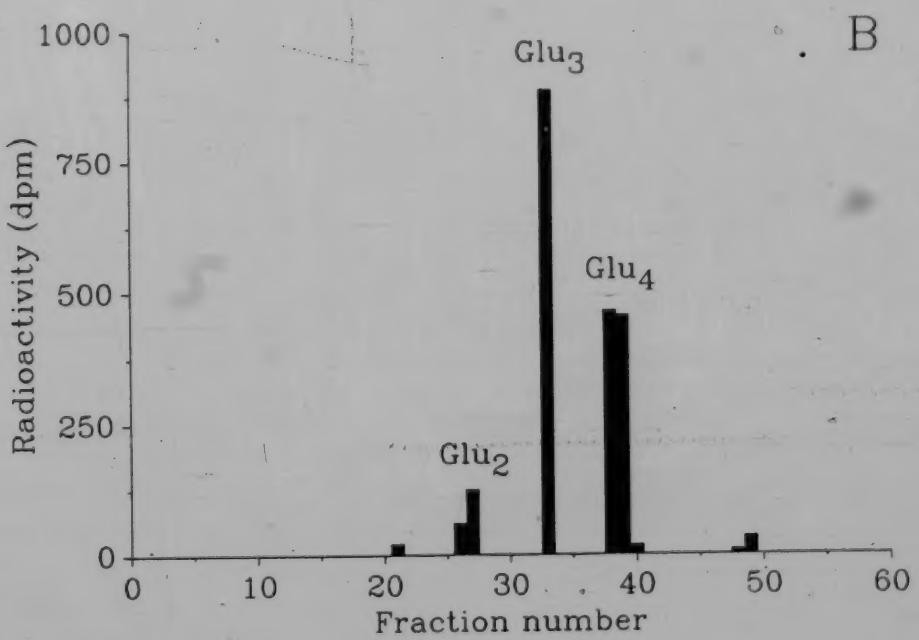
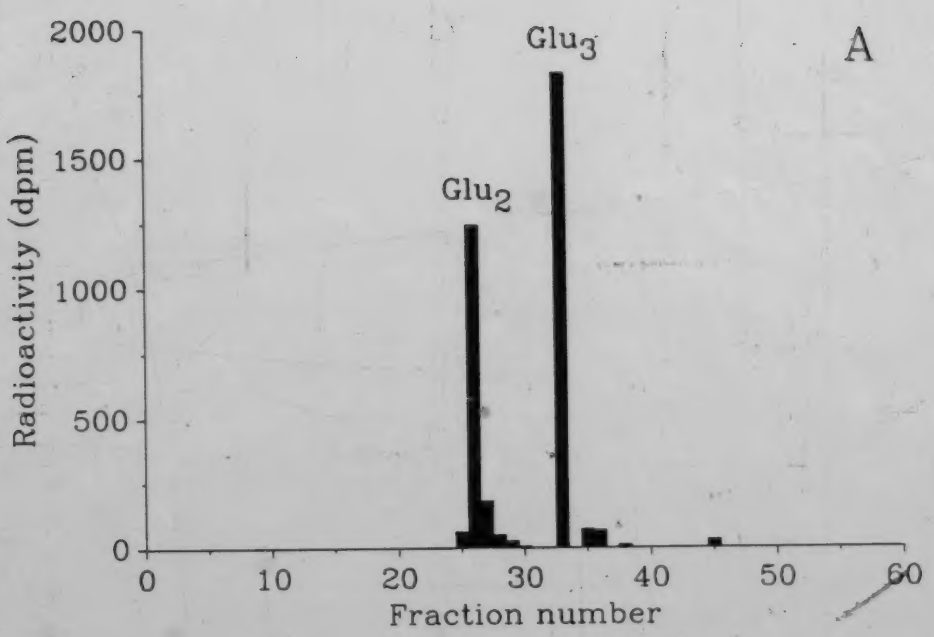

B
1000
750
500
250
0
Radioactivity (dpm)
Glu3
Glu4
Glu2
0
10
20
30
40
50
60
Fraction number

FIGURE 17

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 24 HOUR FPGS ASSAYS USING 50 µM $[R,S]H_4PteGlu$ AS THE SUBSTRATE.

Reaction mixtures containing Step 5 protein and 50 µM $[R,S]H_4PteGlu$ were assayed in triplicate for 24 hours. The resulting HCl washes containing folylpolyglutamates were converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column follwed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent products formed from duplicate assays of FPGS activity.


A
Glu3
Glu2
2000
1500
1000
500
0
Radioactivity (dpm)
0
10
20
30
40
50
60
Fraction number

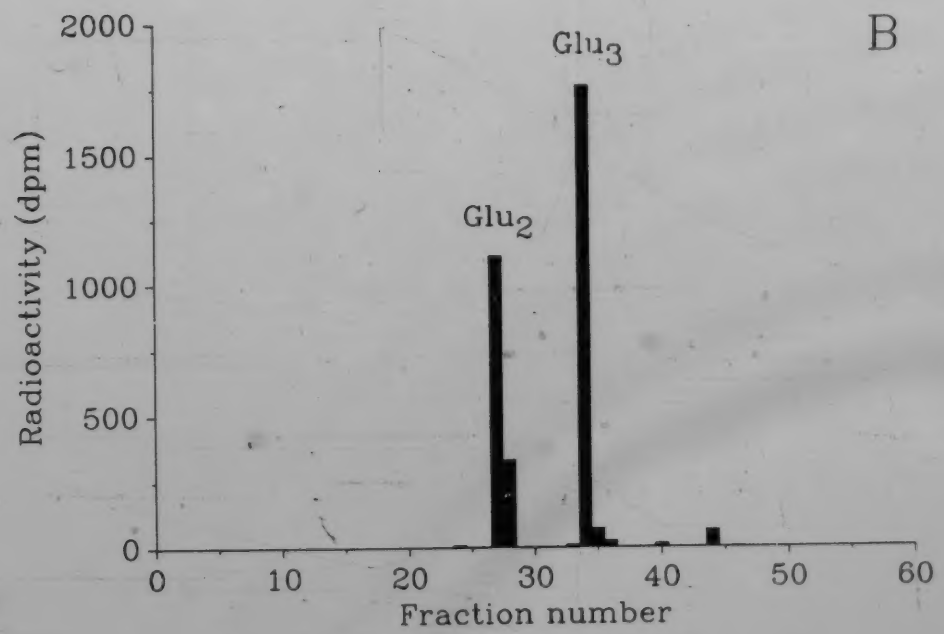
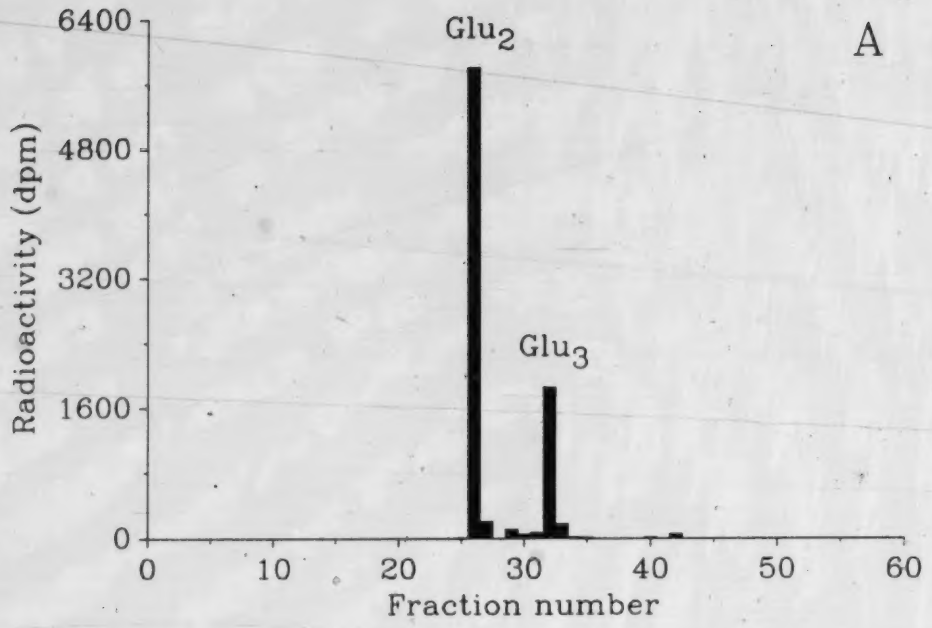

B
Glu3
Glu2
2000
1500
1000
500
0
Radioactivity (dpm)
0
10
20
30
40
50
60
Fraction number

FIGURE 18

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 24 HOUR FPGS ASSAYS USING 100 μM $[R,S]H_4PteGlu$ AS THE SUBSTRATE.

Standard reaction mixtures containing Step 5 protein and 100 μM $[R,S]H_4PteGlu$ were assayed in triplicate for 6 hours. The resulting HCl washes containing folylpolyglutamates were converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent products formed from duplicate assays of FPGS activity.


6400
4800
3200
1600
0
Radioactivity (dpm)
Glu2
Glu3
A
0
10
20
30
40
50
60
Fraction number

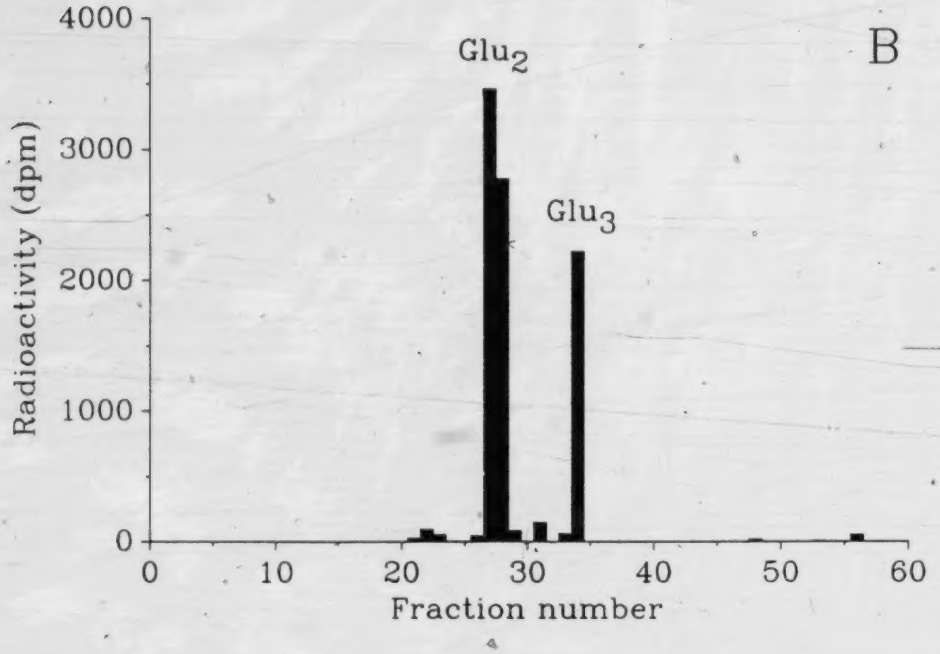
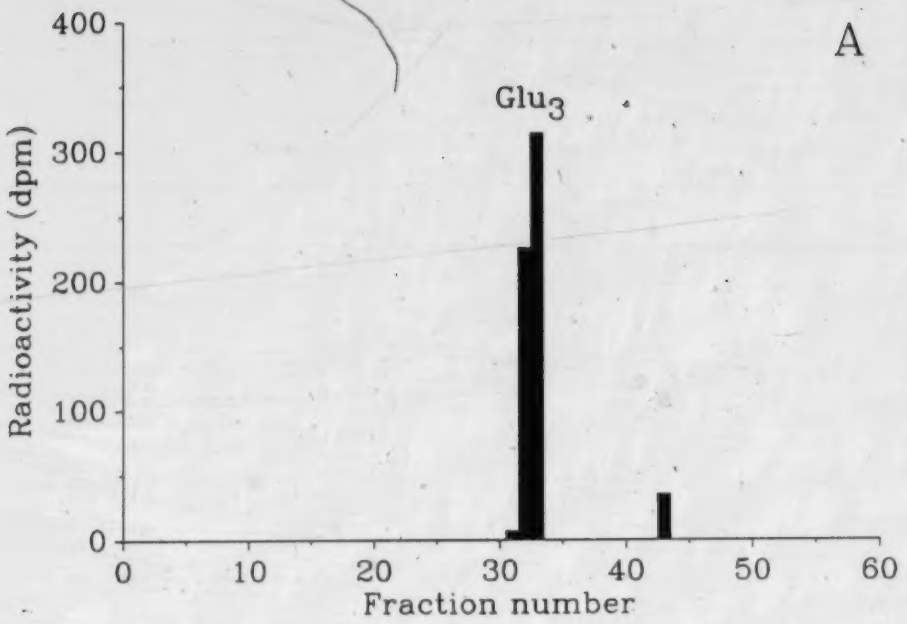

4000
3000
2000
1000
0
Radioactivity (dpm)
Glu2
Glu3
B
0
10
20
30
40
50
60
Fraction number

FIGURE 19

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 2 HOUR FPGS ASSAYS USING 100 μM $[R,S]H_4PteGlu_2$ AS THE SUBSTRATE.

Reaction mixtures containing Step 5 protein and 100 μM [R,S]-$H_4PteGlu_2$ were assayed in triplicate for 2 hours. The resulting HCl washes containing folylpolyglutamates were pooled and converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent duplicate analyses of the pooled HCl wash.


A
Glu3
Radioactivity (dpm)
400
300
200
100
0
0
10
20
30
40
50
60
Fraction number

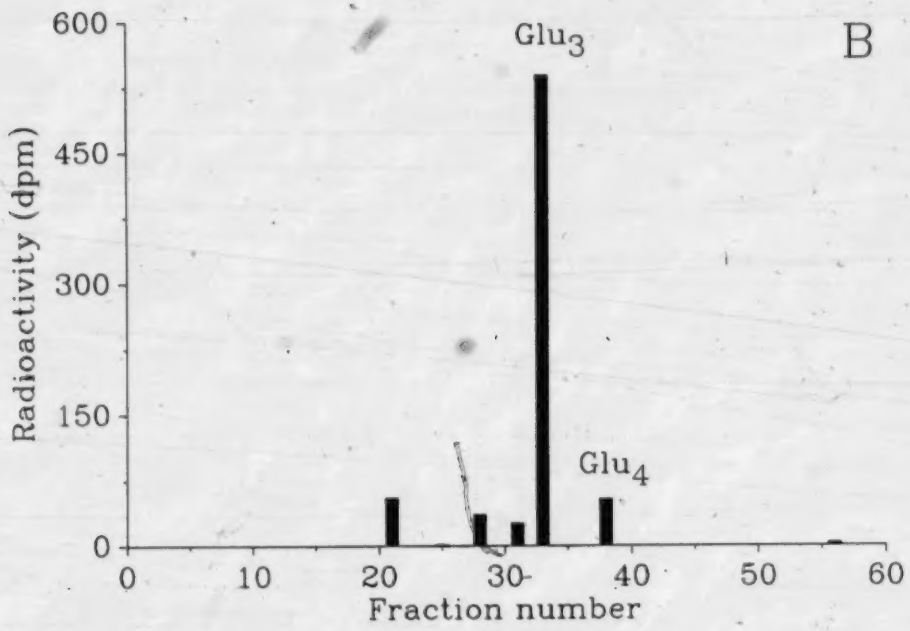
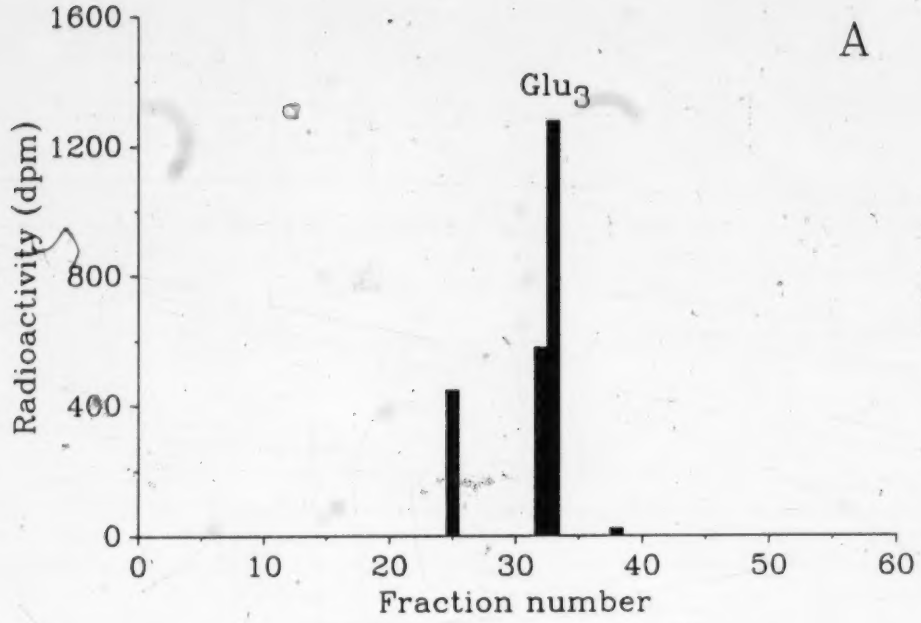

B
Glu3
Glu4
Radioactivity (dpm)
600
450
300
150
0
0
10
20
30
40
50
60
Fraction number

FIGURE 20

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 6 HOUR FPGS ASSAYS USING 100 μM [R,S]$H_4PteGlu_2$ AS THE SUBSTRATE.

Reaction mixtures containing Step 5 protein and 100 μM $H_4PteGlu_2$ were assayed in triplicate for 6 hours. The resulting HCl washes containing folylpolyglutamates were pooled and converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent duplicate analyses of the pooled HCl wash. The unlabelled peak in A does not represent a polglutamate product.


A
Glu3
Radioactivity (dpm)
1600
1200
800
400
0
0
10
20
30
40
50
60
Fraction number

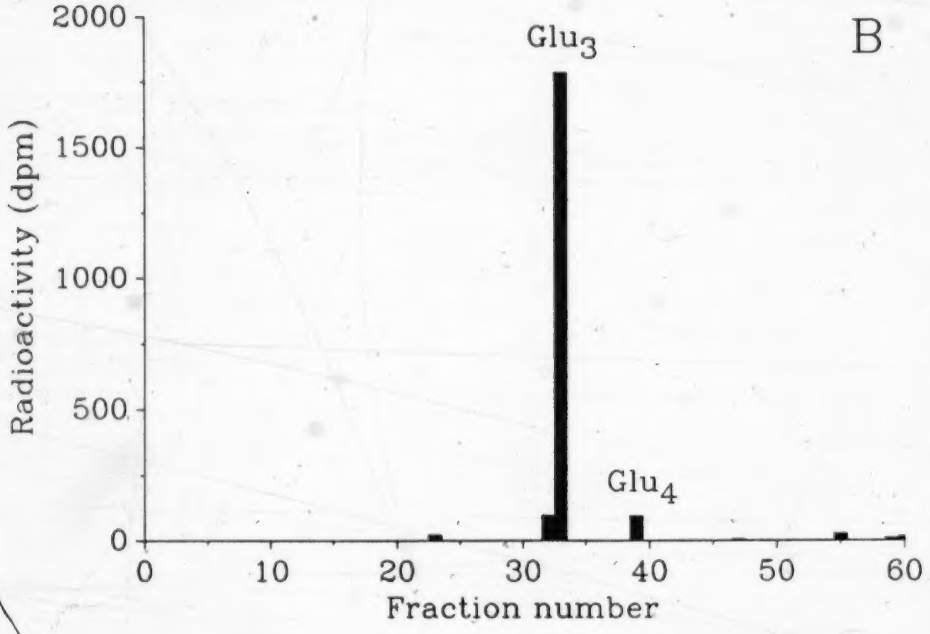
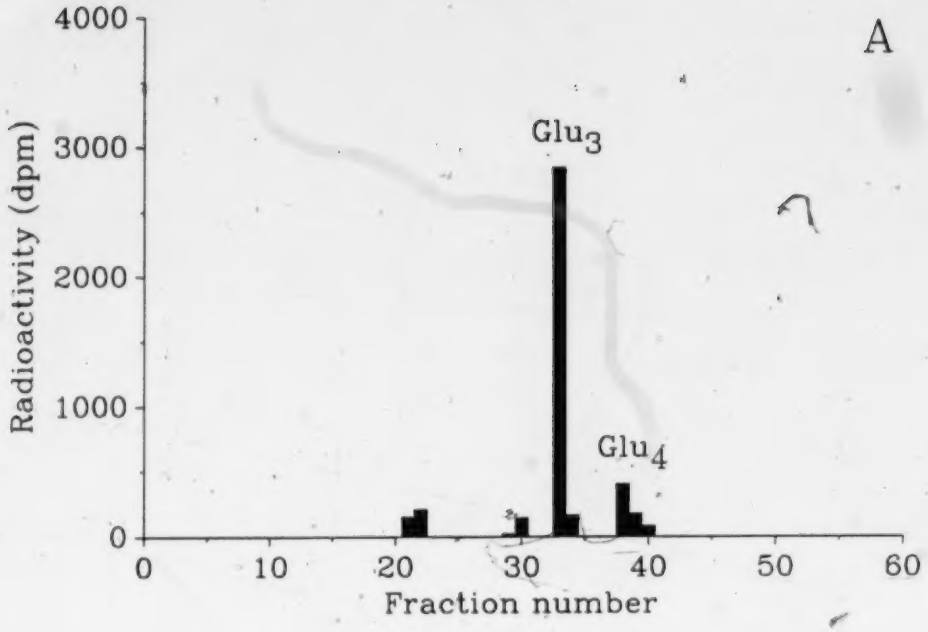

B
Glu3
Glu4
Radioactivity (dpm)
2000
1500
1000
500
0
0
10
20
30
40
50
60
Fraction number

FIGURE 21

HPLC ELUTION PROFILE OF LABELLED $pABAGlu_n$ PRODUCTS DERIVED FROM 24 HOUR FPGS ASSAYS USING 100 µM [R,S]$H_4PteGlu_2$ AS THE SUBSTRATE.

Reaction mixtures containing Step 5 protein and 100 µM $H_4PteGlu_2$ were assayed in triplicate for 24 hours. The resulting HCl washes containing folylpolyglutamates were converted to $pABAGlu_n$ derivatives as described in the Materials and Methods. Chromatography on a strong anion exchange column followed. Fractions of 1 ml were collected and analyzed for radioactivity. A and B represent products formed from duplicate assays of FPGS activity.


A
4000
3000
2000
1000
0
Radioactivity (dpm)
Glu3
Glu4
0
10
20
30
40
50
60
Fraction number

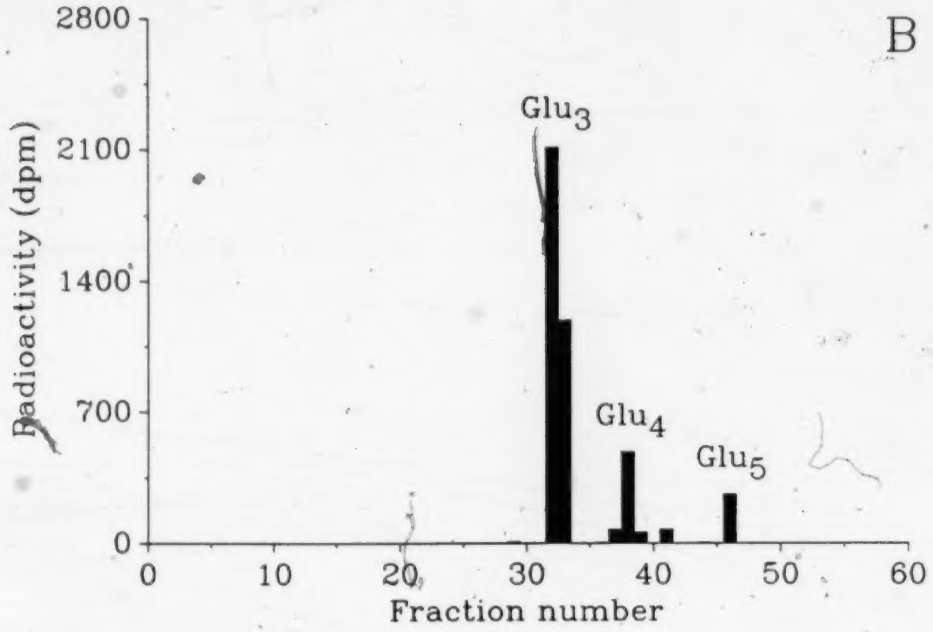

B
2800
2100
1400
700
0
Radioactivity (dpm)
Glu3
Glu4
Glu5
0
10
20
30
40
50
60
Fraction number

Table 10. Characterization of Folate Products of the Pea Cotyledon Polylpolyglutamate Synthetase Reaction.

Treatment Number	Folate Substrate	Time (hr)	Polyglutamate Distribution 2	3	4
1.	100 μM [6 R,S]-H_4PteGlu	2	[a] 9195 [b] (100%) 8910 (92.8)	945 (4.9%)	132.2 (2.3%)
2.	100 μM [6 R,S]-H_4PteGlu	6	16615 (94.8%) 17815 (94.7%)	1725 (4.9%) 2005 (5.3%)	120 (0.2%)
3.	10 μM [6 R,S]-H_4PteGlu	24	1160 (22.9%) 925 (19.7%)	4320 (42.6%) 4435 (47.2%)	5240 (34.5%) 4680 (33.2%)
4.	50 μM [6 R,S]-H_4PteGlu	24	7800 (63.0%) 7220 (60.9%)	9150 (27.0%) 9285 (49.8%)	
5.	100 μM [6 R,S]-H_4PteGlu	24	29970 (85.5%) 31555 (84.7%)	10170 (14.5%) 11390 (15.3%)	
6.	100 μM [6 R,S]-H_4PteGlu$_2$	2		2690 (95.8%) 2690 (94.8%)	175 (4.2%) 220 (5.2%)
7.	100 μM [6 R,S]-H_4PteGlu$_2$	6		9260 (99.2%) 9395 (96.8%)	115 (0.8%) 465 (3.2%)
8.	100 μM [6 R,S]-H_4PteGlu$_2$	24		4995 (87.2%) 16490 (91.4%)	3305 (12.8%) 3090 (8.6%)

The assay conditions and the cleavage of folate products to pABAGlu$_n$ and their separation by HPLC were as described in the Materials and Methods. Folate substrate and incubation time were modified as indicated.

[a] Total dpm incorporated into polyglutamate. Treatments 1, 2, 6 and 7 represent HPLC analyses of three pooled assays. Treatments 3, 4, 5 and 8 are derived from individual assays prepared in triplicate.

[b] (%) distribution of products has been adjusted to account for the increasing specific activity of the folate product with increasing glutamate chain length.

The data presented in Table 10 show that a change in the polyglutamate chain length occurred under the different experimental conditions tested. After a two hour assay period, diglutamates were the predominant chain length formed from H_4PteGlu. Increasing the length of the assay up to 24 hours led to the production of about 15% triglutamate product. However, if the concentration of H_4PteGlu was decreased, an increase in chain length resulted. When only 10 µM H_4PteGlu was used, 47.2% of the product was triglutamate and 33.2% was tetraglutamate. Increasing the concentration to 50 µM led to a loss of tetraglutamate but a considerable amount of triglutamate was still synthesized (39.1%) when compared to the standard (100 µM H_4PteGlu) reaction mixture.

When $H_4PteGlu_2$ was used as the folate substrate, the predominant polyglutamates formed after two, six, or 24 hours were triglutamates. A small amount (8.6%) of tetraglutamate was formed after 24 hours. However, chain lengths longer than the tetra-derivatives were not seen in any of the experimental conditions tested. From other analyses carried out in our laboratory (Imeson et al., submitted for publication), it was determined that the in vivo polyglutamate chain lengths in three day old pea cotyledons were predominantly tetra- and pentapolyglutamates.

IV. DISCUSSION

The discussion presented in the following pages has been organized into six major sections. It begins with a discussion of the distribution of folylpolyglutamate synthetase in plants. Following this, the results obtained from studies of FPGS activity during germination are discussed. The purification of FPGS is then compared with other protocols developed for synthetases from bacterial and mammalian systems. In the fourth section, the properties of the pea cotyledon enzyme are discussed in relation to the properties described for other enzymes studied. A discussion of the nature of the polyglutamate product synthesized by the pea enzyme is then undertaken. The Discussion concludes by pointing out some related questions raised during the course of this research.

4.1 Distribution of Folylpolyglutamate Synthetase

Folylpolyglutamate synthetases isolated from mammalian, bacterial, and fungal sources usually exhibit very low activities (McGuire and Bertino, 1981). Folylpolyglutamate pools are present in all of these organisms, indicating the presence of FPGS in each. However, prokaryotic organisms have 50 to 100 times more activity than eukaryotic organisms (McGuire and Bertino, 1981). Based on the limited survey of plant tissues undertaken in this study (Table 4), folylpolyglutamate synthetase activity was also very low in crude extracts. From the tissues tested, pea cotyledons contained the

greatest amount of activity and were therefore chosen as the source of FPGS in all further studies.

Folylpolyglutamate synthetase activity could not be detected in the wheat and barley leaves examined in this study. However, it is widely acknowledged that plants contain folylpolyglutamates (for a review, see Cossins, 1980), and thus, they must contain FPGS activity. A more complete study of the presence of the enzyme in higher plants should be undertaken. Enzyme activity may have to be concentrated and stabilized to allow its detection, or assays for activity may have to be modified to make them more sensitive. For example, the use of [^{3}H]glutamate with a higher specific activity should promote or increase the detectable level of enzyme activity present.

Although the majority of folylpolyglutamate synthetase activity present in mammals is found in the cytosol, other subcellular activity has been localized in the nucleus, mitochondria, lysosomes and microsomes (McGuire and Coward, 1984). The mitochondrial activity is of particular interest because these organelles contain polyglutamates (Gawthorne and Smith, 1973) and may generate these folates *in situ*. A mitochondrial synthetase has been isolated and examined in *Neurospora crassa* (Cossins and Chan, 1983). The subcellular localization of folylpolyglutamate synthetase has not been examined in higher plants, although current studies in this laboratory have provided evidence for the existence of tetra- and pentaglutamates in pea leaf chloroplasts. Further experiments need to be carried out in which FPGS activities present in plant

mitochondria and chloroplasts are isolated and characterized. Comparisons of these subcellular activities with cytosolic FPGS activity could then be made.

4.2 Folylpolyglutamate Synthetase Activity During Germination

Previous studies have shown that the total folate pool increases rapidly as germination proceeds (Roos and Cossins, 1971; Chan et al., 1986). The folylpolyglutamate content of the cotyledons also increases as the total folate content rises. It is conceivable that the synthesis of polyglutamates is an integral part of the metabolic changes associated with germination as a result of the role they play in the reactions of one-carbon metabolism. Because of this, the activity of folylpolyglutamate synthetase was examined at various stages of germination (Table 5). It is surprising that no appreciable increase in specific activity was observed over the first four days of germination. This suggests that the enzyme is probably present in the mature seed prior to imbibition.

Evidence can be found in the literature suggesting that de novo synthesis of several enzymes occurs during seed germination (Bewley and Black, 1985; Hara and Matsubara, 1980; Nielsen and Liener, 1984; Gifford et al., 1986). Despite this, activity of FPGS was not affected when pea seeds were imbibed in solutions containing cycloheximide or chloramphenicol; inhibitors of cytosolic and organelle protein synthesis, respectively (Table 6). As a result, it seems likely that folylpolyglutamate synthetase in pea seeds is synthesized and "packaged" as the seed matures.

In other studies (Table 6), seeds were imbibed in methotrexate to determine the effect of an inhibitor of tetrahydrofolic acid synthesis on FPGS activity. Methotrexate acts by blocking the activity of dihydrofolate reductase, leading to a build-up of dihydrofolate and thus, a decreased synthesis of THFA. Folylpolyglutamate synthetase activity was not affected by this treatment, indicating that the absence of a folate substrate during germination did not affect the activation of the enzyme.

4.3 Purification of Folylpolyglutamate Synthetase

The protocol used to purify folylpolyglutamate synthetase from pea cotyledons was modelled after the protocol developed by Cichowicz and Shane (1987) in their study of the hog liver enzyme. Other similar methods for bacterial and mammalian synthetases have been developed by Masurekar and Brown (1975), for the E. coli enzyme; Shane (1980), for Corynebacterium; Bognar and Shane (1983), for L. casei; Pristupa et al. (1984), for the beef liver enzyme; and Moran and Colman (1984), for the synthetase from mouse liver. These methods all involved ammonium sulphate fractionation followed by one or more gel filtration, ion exchange, or affinity chromatography steps. All of these protocols attempted to overcome the problem of enzyme stability by the addition of one or more agents to the extracts, including 2-mercaptoethanol, ATP, glycerol, or protease inhibitors.

In the present study, as in the study of the hog liver enzyme (Cichowicz and Shane, 1987), inhibitors of protease activity were added to all buffers to prevent the loss of FPGS activity.

2-Mercaptoethanol was included to provide the necessary reducing conditions. Streptomycin sulphate fractionation prior to treatment with ammonium sulphate enhanced the recovery of folylpolyglutamate synthetase activity in both studies. Pea cotyledon FPGS activity was stabilized in the latter stages of purification by the addition of 50% glycerol. As a result, folylpolyglutamate synthetase activity was purified more than 2700-fold with a yield of approximately 40%. As mentioned in the Results (page 61), this is probably a conservative estimate of purification due to the observation that comparable activities were observed with much lower FPGS concentrations.

4.4 Properties of Folylpolyglutamate Synthetase

The properties of the pea cotyledon folylpolyglutamate synthetase are, in general, similar to those reported for the synthetases from bacterial, mammalian, and fungal systems (Table 11). All of the folylpolyglutamate synthetases examined to date require a folate substrate, MgATP, and L-glutamate for activity. However, the folate substrate preferentially used by these enzymes varies. The Escherichia coli enzyme, for example, utilizes 10-formylH_4PteGlu, while 5,10-methyleneH_4PteGlu is the only effective monoglutamate substrate for the enzyme from Lactobacillus casei (Bognar and Shane, 1983). The Neurospora crassa synthetase also utilizes this folate. Tetrahydrofolate, and in some cases (mouse liver, Moran and Colman, 1984; and beef liver, Pristupa et al., 1984), methylene tetrahydrofolate are used most effectively by mammalian enzymes. The properties of the pea cotyledon synthetase were examined using

Table 11. Physical and catalytic properties of FPGS from various biological sources.

Property	Pea Cotyledon	Hog Liver	Mouse Liver	Rat Liver	Beef Liver	Human Liver
Molecular Weight	68,000	62,000	65,000	69,000	n.d.	n.d.
Folate Substrate	THFA CH_2-folate	THFA	CH_2-folate* CHO-folate**	THFA	CH_2-folate	THFA MTX
pH Optimum	8.5	9.4	>8.5	8.2-8.6	8.4	8.2-8.4
Cation Required	yes	yes	yes	yes	yes	yes
Polyglutamate Product	n=4,5	n=5,6	n=2,4	n=5	n=6	n=2,3
Km Value:						
Folate	3.5 µM	77 µM	3.9 µM	n.d.	9.0 µM	7.0 µM
Glutamate	0.61 µM	0.33 mM	n.d.	0.65 mM	0.82 mM	1.2 mM
ATP	2.31 mM	10 µM	n.d.	70 µM	25 µM	0.25 mM
Reference	This study	Cichowicz and Shane (1987)	Moran and Colman (1984)	McGuire et al. (1980)	Pristupa et al. (1984)	Clarke and Waxman (1987)

n.d. not determined.
* 5,10-methyleneH_4PteGlu
** 10-formylH_4PteGlu

Table 11. Physical and catalytic proterties of FPGS from various biological sources. (Continued).

Property	Coryne-bacterium	L. casei	E.coli	E.coli transformant	CHO cells	N.crassa wild type
Molecular Weight	53,000	43,000	42,000	47,000	n.d.	60,000
Folate Substrate	THFA	CH_2-folate*	CHO-folate	CHO-folate**	THFA	CH_2-folate
pH Optimum	10.0	9.7-10.0	9.0-9.8	n.d.	n.d.	9.8-10.0
Cation Required	yes	yes	yes	yes	yes	yes
Polyglutamate Product	n=2,3	n=3,4	n=2	n.d.	n=4,>4	n=2-6
Km Value:						
Folate	5 µM	2.5 µM	2-12 µM	14.1 µM	n.d.	125 µM
Glutamate	n.d.	0.43 mM	0.18 mM	333 µM	n.d.	n.d.
ATP	n.d.	5600 µM	n.d.	66.5 µM	n.d.	286 µM
Reference	Shane (1980)	Bognar and Shane (1983)	Masurekar and Brown (1975)	Bognar et al (1985)	Taylor and Hanna (1977)	Chan and Cossins (1989)

n.d. not determined
* 5,10-methyleneH_4PteGlu
** 10-formylH_4PteGlu

tetrahydrofolate as the folate substrate. However, from the data in Table 9, it is clear that methyleneH_4PteGlu is also an effective substrate. Other folate derivatives and analogues, including aminopterin, methotrexate, and folic acid, are ineffective as substrates for the reaction.

Examination of other properties reveals that all synthetases have maximal activity at an alkaline pH value. The optimum pH ranges from 8.2 in mammalian livers to around 10 in bacterial and fungal systems. Maximal activity for the pea cotyledon enzyme is found at pH 8.5. Molecular weight determinations show that all enzymes studied are similar in size, although the prokaryotic enzymes are generally somewhat smaller than the enzymes from eukaryotic sources. All enzymes studied to date have an absolute requirement for a monovalent cation with potassium being the most effective (McGuire and Coward, 1984).

The majority of studies undertaken to date have included calculation of the kinetic constants for substrates involved in the folylpolyglutamate synthetase reaction. The Km values for ATP in these reactions range from 0.25 µM with the human liver enzyme to 5600 µM with the enzyme from L. casei. The Km for ATP in the present study was determined to be 2.31 mM using Step 3 (ammonium sulphate fraction) protein. The Km appeared to be much lower when Step 5 protein was added to the reaction. The Km calculated for L-glutamate (0.61 mM) in this study was similar to values determined in other systems, as was the Km for folate (3.5 µM).

The length of the polyglutamate chain produced by the folylpolyglutamate synthetase reaction has been examined in most of the earlier studies (Table 11). A variety of chromatographic techniques have been employed to determine the chain lengths produced. These lengths range from two in E. coli to six or more in hog liver (Cichowicz and Shane, 1987). However, very few of these early studies made use of the sensitive HPLC method developed by Shane (1986). As described previously, conversion of the polyglutamate derivatives to $pABAGlu_n$, followed by formation and purification of the azo dye derivatives is a highly sensitive and accurate means by which polyglutamate chain lengths can be determined. The polyglutamate chain length produced by enzymes of hog liver and pea cotyledons have both been examined by this method. A variety of factors have been shown to influence the production of the polyglutamate chain, including incubation time, and the concentration and type of folate substrate added (Cichowicz and Shane, 1987). These factors and their effect on the synthesis of the polyglutamate chain by pea cotyledon folylpolyglutamate synthetase will be discussed in the next section.

4.5 Nature of the Folylpolyglutamate Synthetase Product

The native polyglutamates of Pisum sativum were initially characterized as 5-methyl and 10-formyltetrahydrofolates (Roos and Cossins, 1971). Recent work has determined that tetra- and pentaglutamates comprise the majority of the native folate pool in the cotyledons, leaves and chloroplasts (Imeson et al., 1989).

Incorporation of [^{14}C]pABA confirmed these findings in the cotyledons and leaves. Imbibition of seeds in methotrexate prior to folate extraction led to an accumulation of this precursor in monoglutamate and short chained polyglutamates. This is clearly consistent with a block in the synthesis of tetrahydrofolate which would indirectly affect polyglutamate synthesis by FPGS.

The synthesis of folylpolyglutamates is affected by substrate concentration and incubation time (Cichowicz and Shane, 1987). In studies of hog liver FPGS, Cichowicz and Shane (1987) determined that physiological concentrations of folate and L-glutamate (0.1 µM and 5 mM respectively) and extended incubation times were required before synthesis of hexaglutamate, the major intracellular form of folate, could occur. In experiments with FPGS from pea cotyledons (Table 10), lowering the concentration of tetrahydrofolate to 10 µM from 100 µM and increasing the length of the incubation resulted in the formation of tri- and tetraglutamates (47.2% and 33.2% of the *in vitro* folate pool, respectively). Pentaglutamates, although comprising the majority of the folate pool *in vivo*, were not recovered as products of the FPGS reaction. It should be noted that [S]H_4PteGlu, the metabolically active isomer of tetrahydrofolate, was not present in physiological concentrations. Calculation of the physiological concentration of H_4PteGlu (based on the data of Chan et al., 1986), gives a value of about 0.18 µM total nonconjugated folate in one day old pea cotyledons. The level may in fact be much lower than this *in vivo* as studies by Roos and Cossins (1971) have

shown that 5-methylH_4PteGlu is a major component of the folate pool of pea cotyledons. Tetrahydrofolate diglutamate was used as the folate substrate in other experiments with FPGS (Table 11). After extended periods of incubation, only small amounts of tetrahydrofolate were synthesized.

4.6 Suggestions for Further Research

Throughout the course of this project, many avenues for further research into this and related areas of plant folate metabolism have been revealed. The points outlined below represent a sampling of interesting questions raised which have yet to be examined and answered in higher plants.

A. Compartmentation of FPGS. The subcellular localization of FPGS has been examined in mammalian and fungal systems. McGuire et al. (1979) showed that rat liver mitochondria have their own synthetase. A mitochondrial synthetase has also been isolated and examined from Neurospora crassa hyphae (Cossins and Chan, 1983). As mentioned previously, studies currently in progress in our laboratory indicate the presence of polyglutamates in pea leaf chloroplasts. However, the isolation and characterization of a plant chloroplast or mitochondrial synthetase has not yet been attempted.

B. Role of conjugase in the turnover of folylpolyglutamates. The role that gamma-glutamyl carboxypeptidases play in the turnover of the folylpolyglutamate pool in higher plants has not been determined. However, it is known that these hydrolases are present in pea cotyledons and other plant tissues in significant amounts (McGuire

and Coward, 1984) and might therefore affect the polyglutamate chain length formed by FPGS.

C. Polyglutamates as substrates in the reactions of C_1 metabolism. Studies of the enzymes catalyzing the reactions of C_1 metabolism in bacterial and mammalian systems have illustrated that polyglutamyl folates act as substrates for many or all of them (reviewed by McGuire and Bertino, 1981). Similar studies in plant systems have yet to be carried out, although this is a basic area of research in the C_1 metabolism of higher plants.

D. Regulation of C_1 metabolism by polyglutamates. Folylpolyglutamates are known to inhibit folate-dependent reactions for which they are not substrates in both bacterial and mammalian systems. Folate metabolism in these systems may thus be regulated to some extent by the relative amounts of substrate and inhibitor present (Kisliuk et al., 1974; Krumdieck et al., 1977; Matthews and Baugh, 1980). Again, equivalent studies in plant systems have not yet been undertaken, although this is a very important and interesting area of research.

The research presented in this thesis will contribute to our knowledge of the folate biochemistry of higher plants. However, the questions raised above also need to be investigated and examined in the years to come in order to develop further insights into the role of plant folates in the reactions of C_1 metabolism.

BIBLIOGRAPHY

ANDREWS, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91: 222-233.

APPLING, D.R. and RABINOWITZ, J.C. 1985a. Regulation of expression of the ADE3 gene for yeast C_1-tetrahydrofolate synthase, a trifunctional enzyme involved in one-carbon metabolism. J. Biol. Chem. 260: 1248-1256.

APPLING, D.R. and RABINOWITZ, J.C. 1985b. Evidence for overlapping active sites in a multifunctional enzyme: immunochemical and chemical modification studies on C_1-tetrahydrofolate synthase from Saccharomyces cerevisiae. Biochemistry 24: 3540-3547.

AVERS, C.J. 1986. Molecular Cell Biology. Addison-Wesley Publishing Co., Ca. pp. 577-579.

BACHMANN, B., HOFMANN, R. and FOLLMANN, H. 1983. Tight coordination of ribonucleotide reduction and thymidylate synthesis in synchronous algae. FEBS Lett. 152: 247-250.

BAGGOTT, J.E. and KRUMDIECK, C.L. 1979. Folylpoly-gamma-glutamates as cosubstrates of 10-formyltetrahydrofolate:5'-phosphoribosyl-5-amino-4-imidazole-carboxamide formyltransferase. Biochemistry 18: 1036-1041.

BAKERMAN, H.A. 1961. A method for measuring the microbiological activity of tetrahydrofolic acid and other labile reduced folic acid derivatives. Anal. Biochem. 2: 558-567.

BASSETT, R., WEIR, D.G. and SCOTT, J. 1976. The identification of hexa, hepta and octaglutamates as the polyglutamyl forms of folate found throughout the growth cycle of yeast. J. Gen. Microbiol. 93: 169-172.

BAUGH, C.M., MAY, L., BRAVERMAN, E. and NAIR, M.G. 1979. Determination of the gammaglutamyl chain lengths in the folates by a combined zinc/acid-peracid procedure. In: Chemistry and Biology of Pteridines. Edited by R.L. Kisliuk and G.M. Brown. Am. Elsevier, New York. pp. 219-224.

BERTINO, J.R. and HILLCOAT, B.L. 1968. Regulation of dihydrofolate reductase and other folate requiring enzymes. Adv. Enz. Regulation 6: 335-349.

BEWLEY, J.D. AND BLACK, M. 1985. Seeds: physiology of development and germination. Plenum Press, New York.

BIANCHETTI, R., LUCCHINI, G., CROSTI, P. and TORTORA, P. 1977. Dependence of mitochondrial protein synthesis initiation on formylation of the initiator methionyl-$tRNA_f$. J. Biol. Chem. 252: 2519-2523.

BLAKLEY, R.L. 1969. The Biochemistry of Folic Acid and Related Pteridines. In: Frontiers of Biology, Vol XIII. Edited by A. Neuberger and E.L. Tatum. Am. Elsevier, New York.

BLAKLEY, R.L. and BENKOVIC, S.J. 1984. Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York.

BOGNAR, A.L., OSBORNE, C., SHANE, B., SINGER, S.C. and FERONE, R. 1985. Folylpoly-gamma-glutamate synthetase-dihydrofolate synthetase. Cloning and high expression of the Escherichia coli folC gene and purification and properties of the gene product. J. Biol. Chem. 260: 5625-5630.

BOGNAR, A.L. and SHANE, B. 1983. Purification and properties of Lactobacillus casei folylpoly-gamma-glutamate synthetase. J. Biol. Chem. 258: 12574-12581.

BRADFORD, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

BRAY, G.A. 1960. A simple and efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1: 279-285.

BRODY, T., SHANE, B. and STOKSTAD, E.L.R. 1979. Separation and identification of pteroylpolyglutamates by polyacrylamide gel chromatography. Anal. Biochem. 92: 501-509.

BRODY, T. and STOKSTAD, E.L.R. 1982. Folate oligoglutamate: amino acid transpeptidase. J. Biol. Chem. 257: 14271-14279.

BROWN, G.M. 1970. Biogenesis and metabolism of folic acid. In: Metabolic Pathways, 3rd ed., Vol. 4. Edited by D.M. Greenberg. Academic Press, New York. pp. 383-410.

CAPERELLI, C.A., BENKOVIC, P.A., CHETTUR, G. and BENKOVIC, S.J. 1980. Purification of a complex catalyzing folate cofactor synthesis and transformylation in de Novo purine biosynthesis. J. Biol. Chem. 255: 1885-1890.

CHAN, P.Y., COFFIN, J.W. and COSSINS, E.A. 1986. In vitro synthesis of pteroylpoly-gamma-glutamates by cotyledon extracts of Pisum sativum L. Plant Cell Physiol. 27: 431-441.

CHAN, P.Y., DALE, P. AND COSSINS, E.A. 1989. Manuscript in preparation.

CHAN, V.T. and BAGGOTT, J.E. 1982. Polyglutamyl folate coenzymes and inhibitors of chicken liver glycinamide ribotide transformylase. Biochim. Biophys. Acta 702: 99-104.

CICHOWICZ, D.J., FOO, S.K. and SHANE, B. 1981. Folylpoly-gamma-glutamate synthesis by bacteria and mammalian cells. Molec. Cell. Biochem. 9: 209-228.

CICHOWICZ, D.J. and SHANE, B. 1987. Mammalian folylpoly-gamma-glutamate synthetase. 1. Purification and general properties of the hog liver enzyme. Biochemistry 26: 504-512.

CLANDININ, M.T. and COSSINS, E.A. 1972. Localization and interconversion of tetrahydropteroylglutamates in isolated pea mitochondria. Biochem. J. 128: 29-40.

CLARKE, L. and WAXMAN, D.J. 1987. Human liver folylpolyglutamate synthetase: biochemical characterization and interactions with folates and folate antagonists. Arch. Biochem. Biophys. 256: 585-596.

COFFIN, J.W. 1985. Folate pools and methionyl-tRNA transformylase activity of plant mitochondria. Possible roles in organelle protein synthesis during germination and early growth of pea and peanut. Ph.D. Thesis, University of Alberta, Edmonton, Canada.

COFFIN, J.W. and COSSINS, E.A. 1986. Mitochondrial folates and methionyl-tRNA transformylase activity during germination and early growth of seeds. Phytochemistry 25: 2481-2487.

COSSINS, E.A. 1980. One-carbon metabolism. In: The Biochemistry of Plants. Vol. 2. Edited by D.D. Davies. Academic Press, New York. pp. 365-418.

COSSINS, E.A. 1984. Folates in biological materials. In: Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 1-59.

COSSINS, E.A. 1987. Folate biochemistry and the metabolism of one-carbon units. In: The Biochemistry of Plants. Vol. 11. Edited by D.D. Davies. Academic Press, New York. pp. 317-353.

COSSINS, E.A. and CHAN, P.Y. 1983. Folylpolyglutamate synthetase activities of Neurospora crassa: nature of the products formed by soluble and particulate enzymes in the wild-type and polyglutamate-deficient mutants. In: Folyl and Antifolyl Polyglutamates. Advances in Experimental Medicine and Biology. Vol. 163. Edited by I.D. Goldman, B.A. Chabner and J.R. Bertino. J.R. Plenum, New York. 163: 183-197.

COSSINS, E.A. and CHAN, P.Y. 1984. Folylpolyglutamate synthetase activities of Neurospora. Phytochemistry 23: 965-971.

CROSTI, P. 1974. Intracellular distribution of 10-formyl tetrahydrofolic acid synthetase in spinach leaves. Ital. J. Biochem. 23: 72-86.

CURTHOYS, N.P., SCOTT, J.M. and RABINOWITZ, J.C. 1972. Folate coenzymes of Clostridium acidi-urici. The isolation of (l)-5,10-methenyltetrahydropteroyltriglutamate, its conversion to (l)-tetrahydropteroyltriglutamate and (l)-10-[^{14}C]formyltetrahydropteroyltriglutamate, and the synthesis of (l)-10-formyl-[6,7-$^{3}H_2$]tetrahydropteroyltriglutamate and (l)-[6,7-$^{3}H_2$]-tetrahydropteroyltriglutamate. J. Biol.Chem. 247: 1959-1964.

DANCHIN, A. 1973. Does formylation of initiator tRNA act as a regulatory signal in E. coli? FEBS Lett. 34: 327-332.

DAVIES, D.D. and CORBETT, R.J. 1969. Glyoxylate decarboxylase activity in higher plants. Phytochemistry 8: 529-542.

DAY, P.L., MIMS, V., TOTTER, J.R., STOKSTAD, E.L.R., HUTCHINGS, B.L. and SLOANE, N.H. 1945. The successful treatment of vitamin M deficiency in the monkey with highly purified Lactobacillus casei factor. J. Biol. Chem. 157: 423-424.

DE MATA, Z.S. and RABINOWITZ, J.C. 1980. Formyl-methenyl-methylenetetrahydrofolate synthetase (combined) from yeast. Biochemical characterization of the protein from an ade3 mutant lacking the formyltetrahydrofolate synthetase function. J. Biol. Chem. 253: 4245-4253.

DIXON, M. and WEBB, E.C. 1964. Enzymes. 2nd ed. Academic Press Inc. New York. pp. 54-166.

FELLER, W., SCHIMPFF-WEILAND, G. and FOLLMANN, H. 1980. Deoxyribonucleotide biosynthesis in synchronous algae cells. Eur. J. Biochem. 110: 85-92.

FERONE, R. and WARSKOW, A. 1983. Co-purification of dihydrofolate synthetase and N^{10}formyltetrahydropteroyldiglutamate synthetase from E. coli. Adv. Exptl. Med. Biol. 163: 167-181.

FOO, S., CICHOWICZ, D.J. and SHANE, B. 1980. Cleavage of naturally occurring folates to unsubstituted p-aminobenzoylpoly-gamma-glutamates. Anal. Biochem. 107: 109-115.

FOO, S.K., MCSLOY, R.M., ROUSSEAU, C. and SHANE, B. 1982. Folate derivatives in human cells: studies on normal and 5,10-methylene tetrahydrofolate reductase-deficient fibroblasts. J. Nutr. 112: 1600-1608.

FOO, S.K. and SHANE, B. 1982. Regulation of folylpoly-gamma-glutamate synthesis in mammalian cells. In vivo and in vitro synthesis of pteroylpoly-gamma-glutamates by Chinese hampster ovary. J. Biol. Chem. 257: 13587-13592.

FRIEDEN, E.H., MITCHELL, H.K. and WILLIAMS, R.J. 1944. Folic acid. II. Studies on adsorption. J. Am. Chem. Soc. 66: 269-271.

GALIVAN, J., BALINSKA, M., NIMEC, Z. and FOX, M.P. 1982. Factors controlling the concentrations of methotrexate in cultured hepatic cells. Adv. Enzyme Regul. 20: 111-121.

GAWTHORNE, J.M. and SMITH, R.M. 1973. The synthesis of pteroylpolyglutamates by sheep liver enzymes in vitro. Biochem. J. 136: 295-301.

GIFFORD, D.J., IMESON, H.C., THAKORE, E. AND BEWLEY, J.D. 1986. Hydrolysis of crystalloid storage protein in castor bean seed endosperm during and following germination. J. Exp. Bot. 37: 1879-1886.

GIOVANELLI, J., MUDD, S.H. and DATKO, A.H. 1980. Sulfur amino acids in plants. In: The Biochemistry of Plants. Vol. 5. Edited by B.J. Miflin. Academic Press, New York. pp. 453-505.

GIOVANELLI, J., MUDD, S.H. and DATKO, A.H. 1985. In vivo regulation of de novo methionine biosynthesis in a higher plant (Lemna). Plant Physiol. 77: 450-455.

GRIFFIN, M.J. and BROWN, G.M. 1964. The biosynthesis of folic acid. III. Enzymatic formation of dihydrofolic acid from dihydropteroic acid and of tetrahydropteroylpolyglutamic acid compounds from tetrahydrofolic acid. J. Biol. Chem. 239: 310-316.

GROBNER, P. and LOIDL, P. 1983. Thymidylate synthetase during proliferation and differentiation of Physarum polycephalum. In: Chemistry and Biology of Pteridines. Edited by J.A. Blair. de Gruyter, Berlin. pp. 717-721.

HALLIWELL, B. 1973. The role of formate in photorespiration. Biochem. Soc. Trans. 1: 1147-1150.

HARA, I. AND MATSUBARA, H. 1980. Pumpkin (Cucurbita sp.) seed globulin VI. Proteolytic activities appearing in germinating cotyledons. Plant Cell Physiol. 21: 233-245.

HIATT, A.J. 1965. Formic acid activation in plants. I. Purification, properties and distribution of formyltetrahydrofolate synthetase. Plant Physiol. 40: 184-188.

HOGAN, A.G. and PARROTT, E.M. 1940. Anemia in chicks caused by a vitamin deficiency. J. Biol. Chem. 132: 507-517.

HUTCHINGS, B.L., STOKSTAD, E.L.R., BOHONOS, N. and SLOBODKIN, N.H. 1944. Isolation of a new Lactobacillus casei factor. Science 99: 371.

IKEDA, M. and IWAI, K. 1970. Biosynthesis of folic acid compounds in plants. VI. The occurrence and properties of the dihydrofolate-synthesizing enzyme in pea seedlings. Plant Cell Physiol. 11: 639-656.

IMESON, H.C., ZHENG, L. AND COSSINS, E.A. 1989. Folylpolyglutamate derivatives of Pisum sativum L. Determination of polyglutamate chain lengths by high performance liquid chromatography following conversion to p-aminobenzoylpolyglutamates. Submitted for publication to Plant Cell Physiol.

IWAI, K., FUJISAWA, Y. and SUZUKI, N. 1972. The accumulation of 5 phosphoribosyl-5-amino-4-imidazolecarboxamide in folate deficient pea seedlings and the enzymatic reaction in which the compound is involved. Agric. Biol. Chem. 36: 398-408.

IWAI, K. and OKINAKA, O. 1968. The biosynthesis of folic acid compounds in plants. II. Some properties of dihydropteroate-synthesizing enzyme in pea seedlings. J. Vitaminol. 14: 170-177.

IWAI, K., OKINAKA, O. and SUZUKI, N. 1968. The biosynthesis of folic acid compounds in plants. I. Enzymatic formation of dihydropteroic acid and dihydrofolic acid from 2-amino-4-hydroxy-6-substituted pteridines by cell-free extracts of pea seedlings. J. Vitaminol. 14: 160-169.

IWAI, K., SUZUKI, N. and MIZOGUCHI, S. 1967. The distribution of formyltetrahydrofolate synthetase in plants, and the purification and properties of the enzyme from pea seedlings. Plant Cell Physiol. 8: 307-325.

KERESZTESY, J.C., RICKES, E.L. and STOKES, J.L. 1943. A new growth factor for Streptococcus lactis. Science 97: 465.

KEYS, A.J. 1980. Synthesis and interconversion of glycine and serine. In: The Biochemistry of Plants. Vol. 5. Edited by T. Miflin. Academic Press, New York. pp. 359-374.

KIKUCHI, G. 1973. The glycine cleavage system: composition, reaction mechanism, and physiological significance. Mol. Cell. Biochem. 1: 169-187.

KISLIUK, R.L. 1981. Pteroylpolyglutamates. Molec. Cell. Biochem. 39: 331-345.

KISLIUK, R.L., GAUMONT, Y. and BAUGH, C.M. 1974. Polyglutamyl derivatives of folate as substrates and inhibitors of thymidylate synthetase. J. Biol. Chem. 249: 4100-4103.

KOZLOFF, L.M. 1980. Folyl polyglutamate and folate-requiring enzymes as bacteriophage T4D baseplate structural components. BioSystems 12: 239-247.

KRUMDIECK, C.L., CORNWELL, P.E., THOMPSON, R.W. and WHITE, W.E., Jr. 1977. Folic acid. Biochemistry and physiology in relation to the human nutrition requirement. National Academy of Sciences, Washington, D.C., pp. 35-42.

LUCCHINI, G. and BIANCHETTI, R. 1980. Initiation of protein synthesis in isolated mitochondria and chloroplasts. Biochim. Biophys. Acta 608: 54-61.

MACKENZIE, R.E. 1984. Biogenesis and interconversion of substituted tetrahydrofolates. In: Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 255-306.

MASUREKAR, M. and BROWN, G.M. 1975. Partial purification and properties of an enzyme from Escherichia coli that catalyzes the conversion of glutamic acid and 10-formyltetrahydropteroyl-glutamic acid to 10-formyltetrahydropteroyl-gamma-glutamylglutamic acid. Biochemistry 14: 2424-2430.

MATTHEWS, R.G. 1984. Methionine biosynthesis. In: Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 497-553.

MATTHEWS, R.G. and BAUGH, C.M. 1980. Interactions of pig liver methylenetetrahydrofolate reductase with methylenetetrahydropteroylpolyglutamate substrates and with dihydropteroylpolyglutamate inhibitors. Biochemistry 19: 2040-2045.

MATTHEWS, R.G., ROSS, J., BAUGH, C.M., COOK, J.D. and DAVIS, L. 1982. Interactions of pig liver serine hydroxymethyltransferase with methyltetrahydropteroylpolyglutamate inhibitors and with tetrahydropteroylpolyglutamate substrates. Biochemistry 21: 1230-1238.

MCBURNEY, M.W. and WHITMORE, G.F. 1974. Isolation and biochemical characterization of folate deficient mutants of Chinese hampster cells. Cell 2: 173-182.

MCGUIRE, J.J. and BERTINO, J.R. 1981. Enzymatic synthesis and function of folylpolyglutamates. Molec. Cell. Biochem. 38: 19-48.

MCGUIRE, J.J. and COWARD, J.K. 1984. Pteroylpolyglutamates: biosynthesis, degradation, and function. In: Folates and Pterins. Vol. 1. Chemistry and Biology of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 135-190.

MCGUIRE, J.J., HSIEH, P., COWARD, J.K. and BERTINO, J.R. 1980. Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. J. Biol. Chem. 255: 5776-5788.

MCGUIRE, J.J., KITAMOTO, Y., HSIEH, P., COWARD, J.K. and BERTINO, J.R. 1979. Characterization of mammalian folylpolyglutamate synthetases. In: Chemistry and Biology of Pteridines. Edited by R.L. Kisliuk and G.M. Brown. Elsevier-North Holland, New York. pp. 471-476.

MITCHELL, H.K. 1944. Folic acid IV. Absorption spectra. J. Am. Chem. Soc. 66: 274-278.

MITCHELL, H.K., SNELL, E.E. and WILLIAMS, R.J. 1941. The concentration of "folic acid". J. Am. Chem. Soc. 63: 2234.

MITCHELL, H.K., SNELL, E.E. and WILLIAMS, R.J. 1944. Folic acid. I. Concentration from spinach. J. Am. Chem. Soc. 66: 267-268.

MITCHELL, H.K. and WILLIAMS, R.J. 1944. Folic acid. III. Chemical and physiological properties. J. Am. Chem. Soc. 66: 271-274.

MITCHELL, M.K., REYNOLDS, P.H.S. and BLEVINS, D.E. 1986. Serine hydroxymethyltransferase from soybean root nodules. Plant Physiol. 81: 553-557.

MITSUDA, H., SUZUKI, Y., TADERA, L. and KAWAI, F. 1966. Biochemical studies of pteridines in plants. II. Biogenesis of folic acid in green leaves: enzymatic synthesis of dihydropteroic acid from guanosine compounds and mechanism of the synthetic pathway. J. Vitaminol. 12: 192-204.

MORAN, R.G. and COLMAN, P.D. 1984. Mammalian folylpolyglutamate synthetase: partial purification and properties of the mouse liver enzyme. Biochemistry 23: 4580-4589.

MORAN, R.G., WERKHEISER, W.C. and ZAKRZEWSKI, S.F. 1976. Folate metabolism in mammalian cells in culture. I. Partial characterization of the folate derivatives present in L1210 mouse leukemia cells. J. Biol. Chem. 251: 3569-3575.

NAKAMURA, K. and KOZLOFF, L.M. 1978. Folate polyglutamates in T4D bacteriophage and T4D-infected Escherichia coli. Biochim. Biophys. Acta 540: 313-319.

NIELSEN, E. and CELLA, R. 1988. Thymidylate synthase in plant cells: kinetic and molecular properties of the enzyme from Daucus carota L. cell cultures. Plant Cell Physiol. 29: 503-508.

NIELSEN, S.S. AND LIENER, I.E. 1984. Degradation of the major storage protein of Phaseolus vulgaris during germination. Plant Physiol. 74: 494-498.

NOUR, J.M. and RABINOWITZ, J.C. 1988. Purification and structural organization of 10-formyltetrahydrofolate synthetase from spinach leaves. J. Cell Biol. 107: 179a.

PAUKERT, J.L., D'ARI-STRAUS, L. and RABINOWITZ, J.C. 1976. Formyl-methenyl-methlenetetrahydrofolate synthetase (combined). An ovine protein with multiple catalytic activities. J. Biol. Chem. 251: 5104-5111.

PAUKERT, J.L., WILLIAMS, G.R. and RABINOWITZ, J.C. 1977. Formyl-methenyl-methylenetetrahydrofolate synthetase (combined): correlation of enzymic activities with limited proteolytic degradation of the protein from yeast. Biochem. Biophys. Res. Commun. 77: 147-154.

PFIFFNER, J.J., BINKLEY, S.B., BLOOM, E.S., BROWN, R.A., BIRD, O.D., EMMETT, A.D., HOGAN, A.G. and O'DELL, B.L. 1943. Isolation of the antianemia factor (Vitamin Bc) in crystalline form from liver. Science 97: 404-405.

PFIFFNER, J.J., CALKINS, D.G., BLOOM, E.S. and O'DELL, B.L. 1946. On the peptide nature of vitamin Bc conjugate from yeast. J. Am. Chem. Soc. 68: 1392.

PRIEST, D.G., DOIG, M.T., BEDNAREK, J.M., MCGUIRE, J.J. and BERTINO, J.R. 1981. Electrophoretic assay of folylpolyglutamate synthetase activity. J. Biochem. Biophys. Methods 5: 273-277.

PRISTUPA, Z.B., VICKERS, P.J., SEPHTON, G.B. and SCRIMGEOUR, K.G. 1984. Folylpolyglutamate synthetase from beef liver: assay, stabilization, and characterization. Can. J. Biochem. Cell Biol. 62: 495-506.

RABINOWITZ, J.C. 1983. Chemical synthesis of folylpolyglutamates, their reduction to tetrahydro derivatives, and their activity with yeast C_1-THF synthase. In: Folyl and Antifolyl Polyglutamates. Advances in Experimental Medicine and Biology. Vol. 163. Edited by I.D. Goldman, B.A. Chabner and J.R. Bertino. J.R. Plenum, New York. pp. 75-83.

RAO, D.N. and RAO, N.A. 1982. Purification and regulatory properties of mung bean (Vigna radiata L.) serine hydroxymethyltransferase. Plant Physiol. 69: 11-18.

RATNAM, S., DELCAMP, T.J., HYNES, J.B. AND FREISHEIM, J.H. 1987. Purification and characterization of dihydrofolate reductase from soybean seedlings. Arch. Biochem. Biophys. 255: 279-287.

REDDY, G.P.V. 1982. Catalytic function of thymidylate synthase is confined to S phase due to its association with replitase. Biochem. Biophys. Res. Commun. 109: 908-915.

REDDY, V.A. and RAO, N.A. 1976. Dihydrofolate reductase from soybean seedlings. Characterization of the enzyme purified by affinity chromatography. Arch. Biochem. Biophys. 174: 675-683.

REID, V.E. and FRIEDKIN, M. 1973. Thymidylate synthetase in mouse erythrocytes infected with Plasmodium berghei. Mol. Pharmcol. 9: 74-80.

RICHARDSON, R.E., HEALY, M.J. and NIXON, P.F. 1979. Folates in rat tissue. Bioassay of tissue folylpolyglutamates and a relationship of liver folylpolyglutamates to nutritional folate sufficiency. Biochim. Biophys. Acta 585: 128-133.

RITARI, S.J., SAKAMI, W., BLACK, C.W. and RZEPKA, J. 1973. The synthesis of polyglutamate forms of folate by N. crassa. Neurospora Newsl. 20: 26-27.

RITARI, S.J., SAKAMI, W., BLACK, C.W. and RZEPKA, J. 1975. The determination of folylpolyglutamate synthetase. Anal. Biochem. 63: 118-129.

ROOS, A.J. and COSSINS, E.A. 1971. Pteroylglutamate derivatives in Pisum sativum L. Biosynthesis of cotyledonary tetrahydropteroylglutamates during germination. Biochem. J. 125: 17-26.

ROSS, J., GREEN, J., BAUGH, C.M., MACKENZIE, R.E. and MATTHEWS, R.G. 1984. Studies on the polyglutamate specificity of methylene tetrahydrofolate dehydrogenase from pig liver. Biochemistry 23: 1796-1801.

ROWE, P.B. 1984. Folates in the biosynthesis and degradation of purines. In: Folates and Pterins. Vol. 1. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp 329-344.

SANTI, D.V. and DANENBERG, P.V. 1984. Folates in pyrimidine nucleotide biosynthesis. In: Folates and Pterins. Vol. 1. Chemistry and Biology of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 345-398.

SAROJINI, G. and OLIVER, D.J. 1983. Extraction and partial characterization of the glycine decarboxylase multienzyme complex from pea leaf mitochondria. Plant Physiol. 72: 194-199.

SCHIRCH, L. 1978. Formyl-methenyl-methylenetetrahydrofolate synthetase from rabbit liver (combined). Evidence for a single site in the conversion of 5,10-methylenetetrahydrofolate to 10-formyltetrahydrofolate. Arch. Biochem. Biophys. 189: 283-290.

SCHIRCH, L. 1982. Serine hydroxymethyltransferase. Adv. Enzymol. 53: 83-112.

SCHIRCH, L. 1984. Folates in serine and glycine metabolism. In: Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 399-431.

SCOTT, J.M. and WEIR, D.G. 1976. Folate composition, synthesis and function in natural materials. Clinics in Hematology 5: 547-568.

SCRIMGEOUR, K.G. 1986. Biosynthesis of polyglutamates of folates. Biochem. Cell Biol. 64: 667-674.

SHANE, B. 1980. Pteroylpoly(gamma-glutamate) synthesis by Corynebacterium species. Purification and properties of folylpoly(gamma-glutamate) synthetase. J. Biol. Chem. 255: 5655-5662.

SHANE, B. 1982. High performance liquid chromatography of folates: identification of poly-gamma-glutamate chain lengths of labeled and unlabeled folates. Am. J. Clin. Nutr. 35: 599-608.

SHANE, B. 1986. Identification of folylpoly(gamma-glutamate chain length by cleavage to and separation of p-aminobenzoylpoly(gamma-glutamates). Methods in Enzymol. 122: 323-330.

SHANE, B. and STOKSTAD, E.L.R. 1984. Folates in the synthesis and catabolism of histidine. In: Folates and Pterins. Vol. 1. Chemistry and Biochemistry of Folates. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 433-455.

SHANE, B. and STOKSTAD, E.L.R. 1985. Vitamin B_{12}-folate interrelationships. In: Annual Review of Nutrition. Vol. 5. Edited by R.E. Olson E. Beutler and H.P. Broquist. Annual Reviews Inc., California. pp. 115-141.

SNELL, E.E. and PETERSON, W.H. 1940. Growth factors for bacteria. Additional factors required by certain lactic acid bacteria. J. Bacteriol. 39: 273-285.

SPRONK, A.M. 1971. The occurrence and function of pteroylglutamates in radish cotyledons. Ph.D. Thesis, University of Alberta, Edmonton, Canada.

STABEN, C. and RABINOWITZ, J.C. 1984. Formation of formylmethionyl-tRNA and initiation of protein synthesis. In: Folates and Pterins. Vol. 1. Edited by R.L. Blakley and S.J. Benkovic. Wiley-Interscience, New York. pp. 457-495.

STOKSTAD, E.L.R. 1943. Some properties of a growth factor for Lactobacillus casei. J. Biol. Chem. 149: 573-574.

SUZUKI, N. and IWAI, K. 1970. The occurrence and properties of dihydrofolate reductase in pea seedlings. Plant Cell Physiol. 11: 199-208.

TAN, L.U.L., DRURY, E.J. and MACKENZIE, R.E. 1977. Methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyl-tetrahydrofolate synthetase. A multifunctional protein from porcine liver. J. Biol. Chem. 252: 1117-1122.

TAYLOR, R.T. and HANNA, M.L. 1977. Folate-dependent enzymes in cultured Chinese hampster cells: folylpolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. Arch. Biochem. Biophys. 181: 331-344.

TAYLOR, R.T. and HANNA, M.L. 1979. Folate dependent enzymes in cultured Chinese hamster ovary cells: evidence for mutant forms of folylpolyglutamate synthetase. Arch. Biochem. Biophys. 197: 36-43.

THOMPSON, G.A., DATKO, A.H., MUDD, S.H. and GIOVANELLI, J. 1982. Methionine biosynthesis in Lemna. Studies on the regulation of cystathionine gamma-synthase, o-phosphohomoserine sulfhydrylase and o-acetylserine sulfhydrylase. Plant Physiol. 69: 1077-1083.

TOLBERT, N.E. 1980. Photorespiration. In: The Biochemistry of Plants. Vol. 2. Edited by D.D. Davies. Academic Press, New York. pp. 487-523.

TOTTER, J.R., SHUKERS, C.F., KOLSON, J., MIMS, V. and DAY, P.L. 1944. Studies on the relation between vitamin M, xanthopterin, and folic acid. J. Biol. Chem. 152: 147-155.

VANDIVER, V.V. and FITES, R.C. 1979. Thymidylate synthase activity from Chlamydomonas cells and cultured tissues of Nicotiana, Pinus, and Daucus. Plant Physiol. 64: 668-670.

WHITFIELD, G.D., STEERS, E.J. and WEISSBACH, H. 1970. Purification and properties of 5-methyltetrahydropteroyltriglutamate-homocysteine transmethylase. J. Biol. Chem. 245: 390-401.

WILLS, L. 1931. Treatment of "pernicious anaemia of pregnancy" and "tropical anaemia". Br. Med. J. 1: 1059-1064.